Induction of Neutralizing Antibodies by Recombinant Nucleocapsid Protein (N) of Hantaan Virus: Potentiality and Implications

Kap-Soo Noh, Sun Pyo Hong, Young-Cheol Shin, Sung-Hee Lee, Hyun-Su Kim, Cha-Yong Choi[1], Zhihui[2] Yao, Soo-Ok Kim and Wangdon Yoo

R&D Center, Cheil Jedang Corp., Dokpyong-Ri Majang-Myon Ichon-Kun Kyonggi-Do, Korea
[1]Department of Chemical Technology, Seoul National University, Seoul, Korea
[2]National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China

=국문초록=

한탄바이러스의 유전자 재조합 내피단백질에 의한 중화 항체의 유도

제일제당 (주) 종합연구소, [1]서울대학교 공업화학과, [2]중국약품생물제품검정소

노갑수 · 홍선표 · 신영철 · 이성희 · 김현수 · 최차용[1] · 여지혜[2] · 김수옥 · 유왕돈

한탄바이러스의 내피 단백질 (N)은 이 바이러스에 대한 중요한 항원으로 작용하지만 신증후출혈열 예방과 관련된 작용은 명확히 알려져 있지 않다. 본 연구는 이러한 내피 단백질이 한탄바이러스에 대한 중화 항체를 유도할 수 있는가 하는 관점에서 수행되었다. 한탄바이러스의 내피 단백질을 대장균에서 용해된 형태로 발현하고 이를 단클론 항체를 이용한 면역친화 컬럼으로 분리 정제하였다. 정제된 내피 단백질을 기니픽에 면역하여 항혈청을 얻고 이것의 한탄바이러스에 대한 중화능력을 중화항체 플락 감소법 (plaque reduction neutralization test)을 이용하여 조사한 결과 최고 1:160의 중화능이 있음을 관찰하였다. 이는 한탄바이러스의 내피 단백질이 중화 항체를 유도할 수 있는 epitopes을 가지고 있음을 의미하며 이러한 생각은 본 연구에서 수행한 면역침강법과 N 단백질에 대한 단클론항체를 이용한 면역친화법을 통한 한탄바이러스의 정제 실험 결과에서도 뒷받침되고 있다.
Key Words: Hantaan virus, Neutralizing antibody, Nucleocapsid protein.

INTRODUCTION

Hantaviruses (family *Bunyaviridae*) can be differentiated into four serologically distinct groups, which include Hantaan virus, Seoul virus, Puumala virus, and Prospect Hill virus. Some hantaviruses are associated with Korean haemorrhagic fever (KHF), epidemic haemorrhagic fever (EHF), nephropathia epidemica (NE) and other pathogenic diseases, collectively termed haemorrhagic fever with renal syndrome (HFRS). Hantaan, Seoul, and Puumala viruses are transmitted by *Apodemus agrarius*, *Rattus norvegicus*, and *Clethrionomys glareolus* respectively. *Microtus pensylvanians* is the primary rodent reservoir for Prospect Hill virus [1-6]. In the Balkan area, a *hantavirus* called Belgrade carried by the yellow-necked field mouse (*Apodemus flavicollis*) was reported as an etiologic agent of HFRS with high mortality [7,8]. Recently Four corners or Muerto Canyon virus was identified as the etiological agent of the hantavirus pulmonary syndrome (HPS) with fatal respiratory illness in U.S.A., which is transmitted to hu-

man mainly from the deer mouse, *Peromyscus maniculatus* [9-13]. This recent unexpected hantavirus outbreak with 76% mortality in 1993 in the U.S.A. also makes *hantavirus* an emerging infectious virus in North America, where Prospect Hill virus has not been reported to be associated with human diseases.

Hantaan virus is an enveloped virus whose core particle consists of tripartite single-stranded RNAs (L segment, M segment, S segment) which are complexed with N protein (nucleocapsid protein) [14]. The M segment encodes G1 (70 Kd) and G2 (55 Kd) glycosylated envelope proteins [15, 16]. The L segment encodes the viral polymerase, and the S segment encodes N protein [15,17,18]. Hantaan virus envelope proteins G1 and G2 are believed to be major viral antigens which can induce neutralizing immunity against Hantaan virus infection. A recombinant vaccinia virus encoding both G1 and G2 induced protective immunity against Hantaan virus challenge in animal models. Cell lysates infected with recombinant baculoviruses expressing Hantaan virus envelope proteins were also demonstrated protection, although G1 or G2 individually were not as effective as G1 and G2 together [16,19,20,21].

In general, humoral antibodies elicited by intact virions are rather specific for components on the virion's surface. However previous reports have shown that most acute phase sera from HFRS infection precipitate only the N protein, and only late convalescent phase sera can precipitate G1 and G2 proteins [13,22]. After the immunization with intact Hantaan virions, G1 and G2 specific antibodies are usually overshadowed by the nucleocapsid-specific antibodies [23]. It is relatively difficult to select hybridoma cells which produce G1 and G2 specific antibodies. To circumvent this problem, Arigawa *et al.* used an alternative method in which G1 and G2 antigens were used as sepharose-bound antigens-antibody complexes [23]. Also in our attempts to im-

munize guinea pigs with formalin-inactivated Hantaan viruses, anti-N antibodies appeared as the major antibodies in the resulting animal sera. Therefore these observations suggested that the N protein is a major immunogen at the early phase of hantavirus infection, but its role in the humoral immune protection from HFRS infection remains to be determined.

The *hantavirus* N protein is the major structural antigen, and it induces strong cross-reactive immunity against viruses in hantavirus genus [24, 25]. A recombinant vaccinia virus expressing the N protein protected immunized animal from subsequent Hantaan virus challenge [19-21]. Cell lysates infected with recombinant baculoviruses expressing the N protein also protected animals in the absence of measurable neutralizing antibodies [19, 20]. These data suggest that the N protein induces protection, and that this protection may involve cell-mediated immunity, although the basis for such protection needs further study.

To gain a better understanding of a role of the N protein in Hantaan virus-induced immunogenicity, we have purified the N protein expressed in *E. coli*, and measured the plaque reduction neutralization titers (PRN titers) of animal sera after immunizing animals with the purified antigens. We have also attempted to purify Hantaan viruses with an affinity column of anti-N protein mAb coupled to Sepharose CL-4B, and have successfully purified viral proteins, including the viral envelop protein G 2. In this study we provide a demonstration that the N protein can induce neutralizing antibodies for protection from Hantaan virus infection.

MATERIALS AND METHODS

Precipitation of viral antigens by anti-N antibodies

Supernatants of Vero E6 cell culture infected with Hantaan virus were incubated with serially diluted guinea pig polyclonal anti-N

protein antisera for 1 h with rotary agitation at 4 ℃. Antigen-antibody precipitates were collected by centrifugation at 5000xg for 10 min. Proteins were transferred from SDS-PAGE gel onto nitrocellulose membrane filters as described by manufacturer (Novex). The electrotransferred filters were first incubated with PBS containing 5% skim milk, and subsequently incubated with a monoclonal anti-N protein antibodies. After washing with PBS buffer containing 0.05% Tween-20, the filters were incubated with goat anti-guinea pig immunoglobulin coupled with horse radish peroxidase and developed with the 4-chloro-1-naphtol in 50 mM Tris-Cl (pH 7.2).

Affinity purification of Hantaan viral antigens

Hantaan virus strain 76-118 was propagated in Vero E6 cells using DMEM supplemented with 10% fetal bovine serum. The supernatants of Vero E6 cell cultures infected with virus were collected after centrifugation at 5000x g for 30 min. The resulting supernatants were passed through CNBr-activated Sepharose CL-4B coupled with murine N protein-specific monoclonal antibodies. The column was washed with 10mM Tris-Cl (pH 7.2) containing 0.15 M NaCl and eluted with 0.1M glycine (pH 12) solution. The anti-N protein mAb was prepared from ascitic fluid of Balb/c injected with hybrid cell line HT90-40. HT90-40 was constructed by fusing spleen cells of Hantaan virus-immunized Balb/c and myeloma cells.

Construction of N protein expression plasmid

On the basis of the nucleotide sequence of the S genome of Hantaan virus 76-118 reported by Schmaljohn et al. [18], we designed PCR primers to amplify the entire coding region of the Hantaan virus N protein. Following PCR amplification, DNA fragments of 1.3 kb encoding the N protein with BglII and EcoRI sites at their 5'-ends and 3'-ends, respectively, were prepared. The DNA fragments were purified by agarose gel electrophoresis and Geneclean Kit (Bio 101, Inc., Vita, CA, USA). Induction of Neutralizing Antibodies by Recombinant Nucleocapsid Protein (N) of Hantaan Virus; Potentiality and Implications). The purified DNA fragments were ligated with BamHI and EcoRI-cleaved plasmid pET-3a and transferred into E.coli BL21 (DE3). Recombinant clones containing the N gene were screened, and the plasmid was designated as pET-NP.

Expression and purification of N protein

BL21 (DE3) cells harboring plasmid pET-NP were grown in 1 liter of L broth supplemented with $100\mu g/ml$ of ampicillin. The culture was induced by adding IPTG to final concentration of 0.5 mM, and was grown for additional 4 h. After harvest, cells were resuspended in $50ml$ of TE buffer, and sonicated 4 cycles, 30 sec per cycle, on ice. For complete lysis, the sonicated cells were passed through French Press twice. The cell lysate was centrifuged at 8,000 rpm with Sorvall SS-34 rotor for 40 min, and the supernatant was obtained. After precipitation of the supernatant with ammonium sulfate, the pellet was resuspended in $30ml$ of PBS (pH 8.0) and dialyzed with 4 liter of PBS overnight. The dialyzed sample was applied to an affinity column with anti-N protein mAb coupled to an CNBr-activated Sepharose CL-4B. After extensive washing with the PBS (pH 8.0) the N protein was eluted with 0.1M glycine (pH 12).

Immunization schedule

The N proteins purified from recombinant E. coli were formulated with CFA (Complete Freund's Adjuvant) or ICFA (Incomplete Freund's Adjuvant). For a comparison, "Hantavax" (Lot No. 3011) which is a formalin-inactivated vaccine was used. Three to four guinea pigs were immunized s.c. 3 times with

Table 1. Neutralizing antibody responses of guinea pia[a] sera to Hantaan virus 76-118

Immunogen	Neutralizing antibody titers[c]
Negative control sera[b]	<1: 20
Formalin-inactivated vaccine	1: 40
CFA	<1: 20
CFA+N protein(40μg)	1: 160

[a]For immunization 3 to 4 guinea pigs were used and antisera were pooled for the PRN assay.
[b]For the negative control, PBS was injected into guinea pigs.
[c]PRN titers were expressed as the reciprocal of the highest dilution of sera in reduction of the number of virus plaques by 80% or more.

40μg of N protein for a single dose. The interval between injections was 15 days. For the 1st N protein injection CFA formulation was used, and ICFA formulation was used for the 2nd and the 3rd injections. Fifteen days after the 3rd injections, the animals were bled by cardiac puncture. Antisera of each group were pooled and used for PRN assays.

PRN assay

Neutralizing antibody titers were determined by a serum dilution PRN method employing immunoperoxidase staining. Briefly, serial 2-fold dilutions (1: 20, 1: 40, 1: 80, 1: 160 and 1: 320) of pre- and postimmunized guinea pig sera were mixed with 100 PFU of strain 76-118 virus and incubated at 37℃ for 1 h. Confluent monolayers of Vero-E6 cells grown in 24-well trays (Linbro; Flow laboratories, Inc., McLean, Va.) were inoculated with 0.4ml of the virus-serum mixtures in duplicate and allowed to adsorb for 2 h at 37℃. Inoculated monolayers were then overlaid with media containing either 0.3 or 0.5% methyl cellulose (Fisher Scientific Co., Fair Lawn, NJ) and incubated for 5 to 10 days. After incubation, the semisolid overlay was removed, and the monolayers were washed three times with 0.01 M PBS (pH7.2), and fixed with a 1:10 mixture of 20% paraformaldehyde and absolute methanol for 10 min at room temperature. Monolayers were rinsed again with PBS and then incubated with antiviral rat antiserum for 30 min 37℃. Thereafter,

the monolayers were rinsed repeatedly (three 5-min washes) with PBS and incubated with horseradish peroxidase-labeled goat antibodies to rat immunoglobulin G (Cappel Laboratories, West chester, PA). Finally, 0.05% diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) prepared in PBS containing 0.01% hydrogen peroxide was added and allowed to react for 10 to 20 min at room temperature, after which plaques were counted. PRN antibody titers were expressed as the reciprocal of the highest dilution of serum resulting reduction in the number of virus plaques by 80% or more.

RESULTS

1. Immunoprecipitaton of Hantaan virion particles with anti-N polyclonal antibodies

Supposing that some regions of the N protein are displayed on the surface of Hantaan virion particles and are accessible to antibodies, We mixed culture supernatant of Hantaan virus-infected Vero E6 cells with serially diluted anti-N protein polyclonal antibodies. After incubation at 37℃ for 1 h, we centrifuged the mixtures at 5000xg and the precipitates were analysed with western blot to examine complexes formed. In the absence of anti-N protein specific polyclonal antibodies, no N proteins were detected by western blot with anti-N protein mAb, ht9040 (Fig. 1). But using 4- to 16-fold dilutions of the polyclonal antibodies, precipitates formed which were detected with the anti-N protein mAb.
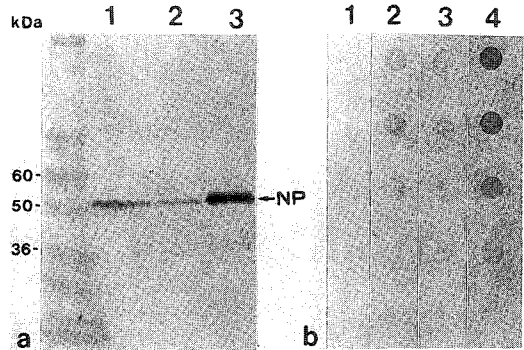
2. Purification of Hantaan virion particles with affinity chromatography of anti-N mAb

Although in this experiment, the complexes formed by anti-N protein specific polyclonal antibodies were efficiently precipitated at low speed of centrifugation, it may still be that only free N proteins were precipitated. Therefore in a second experiment we generated an anti-N protein mAb affinity column and at-
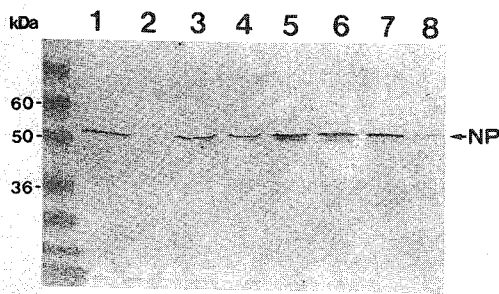
Fig. 1. Western blot analysis of the Hantaan viral proteins which formed Ag-Ab complex with guinea pig polyclonal anti-N protein antisera. 2-fold serially diluted anti-N protein antisera were reacted with the supernatant of virus-infected Vero E6 cell culture. The Ag-Ab precipitates were collected by centrifugation and analyzed by western blot analysis with anti-N protein mAb, ht9040. In lane 1, whole viral particles purified from suckling mouse brain culture through sucrose gradient centrifugation were used as a positive control. Lane 2, no anti-N polyclonal antibodies were added as a negative control. Lane 3-8, Ag-Ab precipitates of Hantaan virus-infected Vero E6 culture supernatant with 1-, 2-, 4-, 8-, 16-, 32-fold serial dilutions of antisera.

Fig. 2. Western and dot blot analyses of immunoaffinity-purified Hantaan viral proteins from Vero E6 cell culture. The murine anti-N protein mAbs(ht9040) were coupled to CNBr-activated sepharose 4B for affinity purification. Supernatant of Vero E6 cell culture infected with Hantaan virus was applied to the affinity column, and eluted with 0.1M glycine buffer, pH 12. The western blot was performed with acute phase HFRS patient sera and dot blot was done with anti-G2 mAb, 48C. A. Western blot analysis of affinity column fractions with HFRS patient sera.Lane 1 represents the supernatant of Vero E6 cell culture infected with Hantaan virus 76-118. Lane2 and 3 represent the flow-through and the eluate on affinity-purification, respectively. B. Dot blot analysis of affinity column fractions with anti-G2 mAb, 48C.The dots in each lane contain 5-fold serially diluted samples. Lane 1 represents the Vero E6 culture supernatant without viral infection. Lane 2, supernatant of virus-infected Vero E6 culture. Lane 3 and 4 represent the flow-through and the eluate, respectively.

tempted to purify Hantaan virions from culture supernatant of Hantaan virus infected Vero E6 cell. By applying the supernatant of the infected Vero E6 cell culture to the anti-N protein-specific mAb affinity column, a peak was obtained with the elution buffer (0.1M glycine, pH 12.0) after extensive washing with buffer containing 10mM Tris, 0.15M NaCl (pH 7.2). The eluate was analysed through a western blot in which acute phase patient anti-Hantaan sera were used to detect the N protein of the eluate. A dot blot analysis was also performed to detect envelope protein G2 of Hantaan virus in the eluate with a conformational epitope-dependent G2 specific monoclonal antibody, 48C. As shown in Figure 2a, the loading material (the culture supernatant of the virus-infected Vero E6 cells) as well as the flow-through showed very weak reactions with acute phase sera in the western blot. But a strong antibody-antigen reaction was seen in

the reaction with the N proteins in the eluate. To determine that we purified not only free N proteins but also the Hantaan virions, we performed a dot blot analysis of 5-fold serially diluted eluate sample with anti-G2 mAb, 48C (Figure 2b). The negative control of culture supernatant of Vero E6 cells without viral infection showed no reaction with anti-G2 mAb, 48C. The loading material and the flow-through fractions in lane 2 and 3 in the Figure 2b showed weak reactions, but in lane 4 the eluate strongly reacted with the anti-G2 mAb up to a 625-fold diluted sample. Thus the data presented in Figure 2a and 2b suggest that the anti-N protein mAb affinity column will purify Hantaan virion particles, and this further sup-
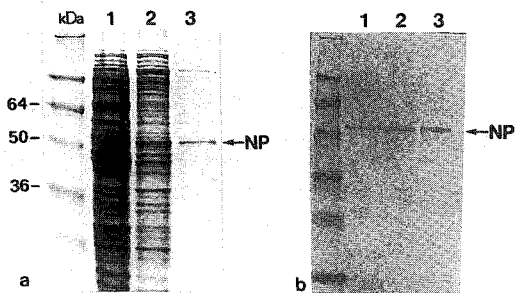
Fig. 3. SDS-PAGE (a) and western blot (b) of the N proteins of Hantaan virus expressed in recombinant *E. coli*. After centrifugation of cell lysate, supernatant (lane 1) was taken, and precipitated with ammonium sulfate. The resulting precipitate was redissolved with PBS, and dialyzed overnight against PBS(lane2). The dialyzed sample was applied to anti-N protein mAb(ht9040)-coupled column. After extensive washing, the loaded sample was pre-eluted with PBS containing 0.3M NaCl, and was eluted with 0.1M glycine buffer(pH12) (lane 3). For the western blot anti-N mAb ht9040 was used.

ports our suggestion that the N protein of Hantaan virion is accessible to the humoral immune system.

3. PRN titers of anti-N sera of guinea pigs immunized with N protein expressed in *E. coli*

Previous reports have suggested that animals immunized with the N protein might be protected from challenge infection with Hantaan viruses, presumably by cell-mediated immune response [19-21]. However in these reports total cell lysate or the recombinant vaccinia virus expressing N protein did not induce neutralizing antibodies. Therefore we decided to determine if purified N protein could induce immunity.

In *E. coli*, the N protein was expressed in both soluble and insoluble forms. This was confirmed by a western blot analysis of supernatant and pellet of the *E. coli* lysates. We purified only soluble form of the N protein to lessen the possibility of incorrect folding or

conformational changes during the purification of insoluble N protein.

The N protein expressed in *E. coli* has a slightly higher molecular weight because of presence of additional 14 amino acids leader which is due to the sequence between the *Bam*HI site and ⌀10 promoter on plasmid pET-3a, and the *Bgl*II site introduced during PCR amplification of N protein gene. The N protein purified through the anti-N protein mAb affinity column chromatography is shown in Figure 3a. The final yield of purified soluble N protein was 0.5 mg to 1.0 mg per liter of culture broth. Purity as assessed by SDS-PAGE was more than 90%. The antigenicity of the purified N protein was confirmed with a western blot using anti-N protein mAb (ht9040) as a primary antibody (Figure 3b). The purified proteins were injected into guinea pigs subcutaneously for 15 days (three injections) and then the animals were bled by cardiac puncture and PRN titers were measured on Vero E6 cells. As summarized in Table 1, the negative control sera without immunization and sera from guinea pigs with CFA alone showed no significant PRN titers (<1: 20) as expected. But sera from animals immunized with formalin-inactivated HFRS vaccines, which were prepared from suckling mice brains, gave 1: 40 of PRN titer after the 3rd immunization. However sera from guinea pigs immunized with 40μg of the purified N protein /dose formulated with CFA showed 1: 160 of PRN titer which is relatively high. Therefore this result provided a direct evidence that the N protein can induce neutralizing antibodies, and suggest that the N protein of a Hantaan virion is accessible to the humoral immune system to induce neutralizing antibodies for protection from Hantaan virus infection.

DISCUSSION

Although the *hantaviruse* in North America

is relatively new, it is no stranger in other parts of the world as causative agents of HFRS. The severity of the disease varies with the geographical locations and the natural hosts of *hantaviruses*. The affected regions include China, Korea and eastern parts of the former Soviet Union. In China and Korea, 100, 000-150,000 cases and 600-1,000 cases of HFRS respectively were annually reported [26, 27]. A number of purified inactivated suckling rat brain vaccines and cell culture-derived vaccines were developed [28-30]. More recently a vaccinia-vectored recombinant vaccine has been developed and evaluated [19].

The N protein, the major structural protein of *hantavirus*, has been reported for its diagnostic value by several studies [24,25,31]. Detection of N-specific antibodies is the major value for the diagnosis of infection with *hantavirus* because sera of HFRS infected patients at early acute phase already have N protein-specific antibodies [13,22]. But the prophylactic value of the N protein is currently unclear. Only the cell-mediated immune response by the N protein has been suggested for a protection of animal from Hantaan virus challenge in the absence of neutralizing antibodies [19,32]. However lysate of cell infected with the baculovirus recombinant expressing the S segment protected all animals but the recombinant vaccinia expressing the S segment protected only some of animals from infection [20,21]; although the live recombinant vaccinia virus is supposedly better to induce the cell-mediated immune response. Thus, the possibility has remained that antibody-mediated immune response by the N protein plays a role for the humoral immune protection.

In our experiments, we expressed the N protein of Hantaan virus in *E. coli* and purified it through an anti-mAb affinity column. With the purified N protein, we clearly demonstrated induction of neutralizing antibodies in guinea pigs after immunization. This observation is rather contradictory to previous reports in which the N protein did not show a measurable PRN titer. The difference may be due to the difference in N-specific antibody titers. In our system we used the purified N protein for immunization but in the previous report crude cell extracts were used. Another possibility might be that the N protein expressed in *E. coli* is more immunogenic than the proteins expressed by vaccinia virus or baculovirus.

How can the nucleocapsid protein in Hantaan virion particles induce neutralizing antibodies? This may be explained by the precipitation study in Figure 1 and the purification study with anti-N protein mAb affinity column in Figure 2. These experiments demonstrated that the virion particles can be precipitated with anti-N protein specific polyclonal antibodies or purified through anti-N protein mAb affinity column. Also examination of the amino acid sequence of the N protein predicted two possible transmembrane helices at amino acids 133 - 157 and 332-349 with buried-helix parameter peak values of 1.23 and 1.248 according to method of Rao and Argos which predicted the transmembrane helix of envelope protein G2 at C-terminus with a peak value of 1.413 (data not shown) [33]. It is possible that the epitopes of the N proteins for neutralizing antibodies are actually displayed on the surfaces of Hantaan virions. The humoral immune response against the N protein in a Hantaan virion is also supported by other studies. Sequencing of S segment of strains within a local rodent population revealed a region (aa 230 to 310) of a genetic drift for the evolution of the virus, but at the protein level a strong stabilization of protein sequence was detected in the region [34]. The most variable region of the N protein of quasispecies is also mapped to the same region that is in turn the most variable region among different serotypes of hantaviruses, suggesting that the N protein could be subject to the humoral immune pressure

and that this region could be a target for neu-tralizing antibodies [18, 35-38].

In *hantavirus* infection, strong N protein and G1 antibody responses appear in the acute phase. G2 antibodies do not appear until the late convalescence. We do not understand mechanisms of neutralization by N protein specific antibodies. A few possible mechan-isms can be only speculated at present. One mechanism could be that N protein specific an-tibodies, presumably group-specific antibodies cooperate with other type-specific antibodies of G1 and G2 to enhance neutralization ac-tivities of humoral immune system. A second mechanism could involve complement medi-ated-immune response which can lyse en-veloped virions. In a study of typing IgG sub-class specific to N protein of HFRS patient sera, we have found that IgG3 is the major subclass at the acute phase (data not shown). In that IgG3 is most efficient for promoting the complement fixation, activation of the com-plement fixation by anti-N protein specific an-tibodies at early stage of infection can play an important role in neutralization of viruses [39].

In conclusion we have demonstrated that an-ti-N protein specific polyclonal antibodies can neutralize Hantaan viruses *in vitro*. The plau-sible explanation is that some epitopes of the N protein in a Hantaan virion are accessible to the immune system, or extracellulary located to induce neutralizing antibodies. These an-tibodies could neutralize the viral infection by cooperating other neutralizing antibodies or enhancing the neutralizing activities. As far as we know this is the first demonstration of neu-tralizing antibody induced by the purified N protein. With this regard, the N protein will be a important antigen for the future development of a recombinant vaccine

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