IMMUNOLOGICAL PROPERTIES OF A FUSION PROTEIN CONTAINING NUCLEOCAPSID PROTEIN AND GLYCOPROTEIN Gn OF HANTAAN VIRUS

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Summary. – *Escherichia coli* and the baculovirus Bac-to-Bac system were used to express chimeric gene GnS0.7 consisting of glycoprotein Gn gene and the 0.7 kb fragment of S genome segment carrying nucleoprotein (N) gene of Hantaan virus (HTNV). The expressed fusion protein GnN0.7 was recognized by monoclonal antibodies (MAbs) to HTNV Gn glycoprotein and N protein, respectively. Its molecular mass as determined by Western blot analysis corresponded to the predicted value. The mice immunized with insect *Spodoptera frugiperda* (Sf9) cells transfected with recombinant baculovirus carrying GnS0.7 gene, produced serum antibodies with titer up to 3,200 as assayed by immunofluorescence. Moreover, immunized mice showed positive proliferation index for splenocytes stimulated with HTNV Gn and N, respectively. These results indicated that insect Sf9 cells infected with the recombinant baculovirus expressed a fully biologically active fusion protein that elicited not only humoral but also cellular immune response in mice. Hence, this protein may be used as a genetically engineered subunit HTNV vaccine representing efficacious and safe alternative to traditional vaccines.

Key words: Hantaan virus; fusion protein; immune response

Introduction

Hantaan virus belongs to the genus *Hantavirus*, family *Bunyaviridae* (Hong and Zhou, 2006). It causes a severe form of hemorrhagic fever with renal syndrome (HFRS), a widespread disease infecting both animals and humans (Lee, 1982; Lee *et al*., 1981). In China, about 50,000 – 100,000 people a year were infected with HTNV with severe symptoms and high mortality. Despite many investigations, a little is known about the pathogenesis of HTNV infection in humans, because of the lack of an appropriate animal model for investigation of this disease. Up till now, there are no specific effective drugs and prophylactic vaccines against this disease.

The genome of HTNV consists of three negative-strand RNA segments, the large (L), medium (M), and small (S). They encode RNA-dependent RNA polymerase (L segment), two surface glycoproteins Gn and Gc (previously referred to as G1 and G2) that are named according to their location relative to the N- and C-terminus of a polyprotein encoded by the M segment, and N protein encoded by the S segment (Schmaljohn, 1990; Schmaljohn *et al*., 1987, 1986). It is well known that neutralizing antibodies especially to Gn and Gc, play a major role in the protection against HTNV infection (Yoshimatsu *et al*.,

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Abbreviations: AcMNPV = Autographa californica multicapsid nucleopolyhedrovirus; CTL = cytotoxic T-lymphocyte; Gc = glycoprotein coming from the carboxy terminus of the polyprotein precursor; Gn = glycoprotein coming from the amino terminus of the polyprotein precursor; GST = glutathione S-transferase; HFRS = hemorrhagic fever with renal syndrome; HTNV = Hantaan virus; IFA = immunofluorescent assay; MAb(s) = monoclonal antibody(ies); N = nucleoprotein; Sf9 cells = *Spodoptera frugiperda* cells

1993; Schmaljohn *et al*., 1990; Zhang *et al*., 1988). Kariwa *et al*. (1995) found that HTNV could replicate or survive for a certain period in adult mice in spite of the presence of specific antibodies. Although a high level of neutralizing antibodies was present in suckling mice inoculated with HTNV, the virus persisted in the mice for several weeks (Nakamura *et al*., 1985). Therefore, it seems that neutralizing antibodies alone could not provide an efficient protection against HTNV infection and need the co-action with the cellular immune system. Yoshimatsu *et al*. (1993) immunized adult mice with recombinant HTNV glycoproteins without eliciting the neutralizing antibodies. However, suckling mice were partially protected by the inoculation of spleen cells from the immunized mice. It suggested that glycoproteins might also play an important role in the cell-mediated protective immune response. Besides, the virulence of HTNV was decreased by a single amino acid change in glycoprotein Gn, what affected the pathogenicity of HTNV as found in the newborn mouse model (Ebihara *et al*., 2000).

The N protein of HTNV is antigenically and genetically more conserved than the envelope glycoproteins. The antigenic sites of N protein are mainly distributed close to the N-terminus (Yoshimatsu *et al*., 1993). Also, our previous study showed that the antigenic sites of N protein were mainly distributed in the 0.7 kb fragment of S segment that corresponds to the N-terminus region (Xu *et al*., 2000, 1992, 1989, 1988). It is known that the N protein could not stimulate the production of neutralizing antibodies, but the protection against HTNV infection could be provided by immunization of mice with N protein or by inoculation of mice with MAbs against N protein (Yoshimatsu *et al*., 1993). Besides, Schmaljohn *et al*. (1990) reported that baculovirus recombinants expressing only N protein could protect mice against HTNV challenge. This outcome suggested that the N protein elicited a non-neutralizing, perhaps T cell-mediated, protective immune response. Several specific cytotoxic T-lymphocyte (CTL) epitopes have been identified on the N protein (Park *et al*., 2000; van Epps *et al*., 1999).

Although HTNV inactivated vaccine has been developed, it has many shortcomings (Cao and Wang, 2008). Nowadays, the effort is concentrated on the development of genetically engineered vaccine and the great emphasis is put on the glycoproteins, because they are able to elicit a production of neutralizing antibodies.

In this study, we examined the expression of fusion protein consisting of the N protein and glycoprotein Gn of HTNV by using a baculovirus expression system (Bac-to-Bac) or a bacterial expression system in *E. coli*. The humoral and cellular immune responses of the expressed protein produced in insect cells were preliminarily characterized to evaluate the possiblity of developing genetically engineered subunit HTNV vaccines.

Materials and Methods

Cells and viruses. Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) and recombinant viruses were propagated and assayed in insect Sf9 cells according to Bac-to-Bac Baculovirus Expression System (Invitrogen). HTNV, strain 76-118 (provided by Prof. G. Song, Chinese Center for Disease Control and Prevention) was propagated in Vero-E6 cells (Schmaljohn *et al*., 1983).

Recombinant plasmid and its expression in E. coli. Gn fragment of M genome segment and 0.7 kb fragment of 5'-end of S genome segment were obtained by PCR. Primers are CMF1 (Gn upstream): 5'-GCGGTACCATGGGG(GA)TATGGAAGTGGCTAGT-3', G1down (Gn downstream): 5'-CAGGTCGACCTTTAGATCAT CCCTG-3', 0.7up (S0.7 upstream): 5'-CAGGTCGACATGGCA ACTATGGAG-3', 0.7down (S0.7 downstream): 5'-GACGAATT CCTCGAGAACTGCTGCTGTATCTGG-3'. GnS0.7 chimeric gene was spliced by putting Gn at the N-terminus of N0.7. The chimeric gene was ligated into a prokaryotic expression vector pGEX-4T2 for producing a recombinant vector pGEX-4T2-GnS0.7, which was transferred into *E. coli* JM109. The expressed protein was fused to the glutathione S-transferase (GST) and signed GST-GnN0.7. The expressed protein was identified by ELISA and Western blot analysis.

Recombinant baculovirus and its expression in Sf9 cells. The recombinant baculovirus containing GnS0.7 chimeric gene was obtained by using the Bac-to-Bac Baculovirus Expression System (Luckow *et al*., 1993). PCR amplification was used to verify the presence of the GnS0.7 gene in the recombinant baculovirus, which was signed as Bac-GnS0.7. Sf9 cells were infected with the amplified Bac-GnS0.7 recombinant and the GnN0.7 protein expression was analyzed by indirect immunofluorescent assay (IFA), ELISA, and Western blot analysis.

Immunization of mice. Sf9 cells were infected with recombinant Bac-GnS0.7 and AcMNPV as the negative control. Three days after the infection, the cells were collected and frozen at -70°C. After 3 cycles of thawing and freezing, the cell lysates were resuspended in PBS followed by mixing with complete Freund's adjuvant. Two groups of 6 Balb/c mice were immunized subcutaneously with equal amounts of AcMNPV- or Bac-GnS0.7-infected cell lysates (10^6 cells/mouse). Mice immunized with non-infected Sf9 cells were used as the negative control. After 2 weeks a booster immunization dose was given subcutaneously with the same amount of antigen mixed with incomplete Freund's adjuvant. Two weeks later, the mice were injected intraperitoneally with the cell lysates. Two weeks after the last injection, the sera from immunized mice were collected and tested for the presence of specific antibodies.

Indirect immunofluorescent assay. Serial dilutions of serum samples were loaded on the acetone-fixed smears of Vero-E6 cells infected with HTNV, strain F_1M_{14} (major epidemic strain in China and with high homology to strain 76-118). FITC-conjugated rabbit anti-mouse IgG was used as the secondary antibody. IFA titers are expressed as the reciprocal of the highest serum dilution that displayed characteristic fluorescence in the cytoplasm of the infected cells.

The fusion protein GnN0.7 expression was analyzed using acetone-fixed smears of Sf9 cells infected with Bac-GnS0.7 or

AcMNPV and incubated with MAb 1A8. Bound antibodies were detected by FITC-conjugated rabbit anti-mouse IgG.

ELISA. The purified HTNV N protein or glycoproteins Gn and Gc (Lanzhou Biological Product Academy, China) were used as coating antigen. The serum specimens were serially diluted. Bound antibodies were detected by horseradish peroxidase (HRP)-labeled rabbit antimouse IgG. and visualized by addition of o-phenylenediamine (OPD) (Xu *et al*., 1992).

Sandwich ELISA. The fusion protein GnN0.7 expression in *E. coli* or Sf9 cells was analyzed by the sandwich ELISA. The microtiter plates were coated with MAb 1A8 (against N protein, prepared in our laboratory), MAb 6F7 (against Gn glycoprotein, provided by Changshou Hang in Chinese Center for Disease Control and Prevention) or Sp2/0 cultivation medium. HRP-labeled MAb 1A8 was used as the secondary antibody and the bound antibodies were detected with OPD. The samples of cell lysates were considered as positive, when the ratio of A_{490} value of sample bound MAbs 1A8 or 6F7 to A_{490} value of sample bound to Sp2/0 was more than 2.1.

Western blot analysis. Proteins were separated in 12% SDS-PAGE and transferred to the nitrocellulose membrane. The membrane with blotted proteins was incubated with HRP -labeled MAb 1A8. Visualization of bound antibodies was performed by addition of 3,3-diaminobenzidine tetrahydrochloride.

Virus neutralization. Series of diluted mouse sera were mixed with 100 $TCID_{50}$ of HTNV (strain F_1M_{14}) and incubated with Vero-E6 monolayer in microtiter plates for 90 mins. Then, the supernatant was discarded and the cells were supplemented with fresh MEM medium. After 9 to 11 days, the infected Vero-E6 cells were frozen and thawed and the presence of HTNV antigen was detected by ELISA. Virus neutralization titer was expressed as the reciprocal of the highest serum dilution that provided a ratio (positive to negative sample) greater than 2.1.

Lymphocyte proliferation assay. 8 weeks after the last immunization, the splenocytes harvested from the immunized mice were plated into 96-well plates and incubated for 3 days with purified N protein or glycoproteins Gn and Gc that were responsible for the induction of cellular response to HTNV. Cell proliferation was measured by the methabenzthiauron (MTT) assay (Hansen *et al*., 1989). After 4 hrs of incubation with MTT, the cells were lysed and the color reaction was measured as $A_{490.}$ The proliferation index was represented as the ratio of the value A_{490} obtained after stimulation of cells to the value A_{490} of non-stimulated cells.

Results

Expression of GST-GnN0.7 fusion protein in E. coli

The prokaryotic expression vector pGEX-4T2 can express the chimeric protein GnN0.7 fused to the GST in *E. coli*. The fusion protein GST-GnN0.7 was recognized by the specific MAbs against N protein or glycoprotein Gn in ELISA. The positive A_{490} to negative A_{490} ratio was more than 10, when fusion protein was detected with MAb 1A8 specific for N protein. On the other hand, the ratio was about 3, when fused protein was detected with MAb 6F7 specific for glycoprotein Gn. The average value (A_{490}) of negative control was 0.06.

In Western blot analysis, we found a novel protein band with M_r more than 100 K that could specifically bind the MAb 1A8 (Fig. 1). The M_r of GST, N0.7, and Gn are about 26 K, 26 K, and 63 K, respectively, what corresponded roughly with the detected M_r of the novel protein band. Therefore, the results of Western blot analysis showed that the fusion protein GST-GnN0.7 recognized by MAb 1A8 was produced as a whole.

Expression of GnN0.7 fusion protein in Sf9 cells

The recombinant baculovirus DNA and AcMNPV DNA were used as the templates for PCR. By using the primers CMF1 and 0.7down, the PCR product was shown to be a fragment of approximately 2.5 kb that corresponded to the entire 1.8 kb HTNV Gn segment along with about 0.7 kb fragment of S segment 5'-end. Insect Sf9 cells infected with recombinant Bac-GnS0.7 were examined by IFA using MAb 1A8. We observed the positive fluorescence in cells 48 hrs

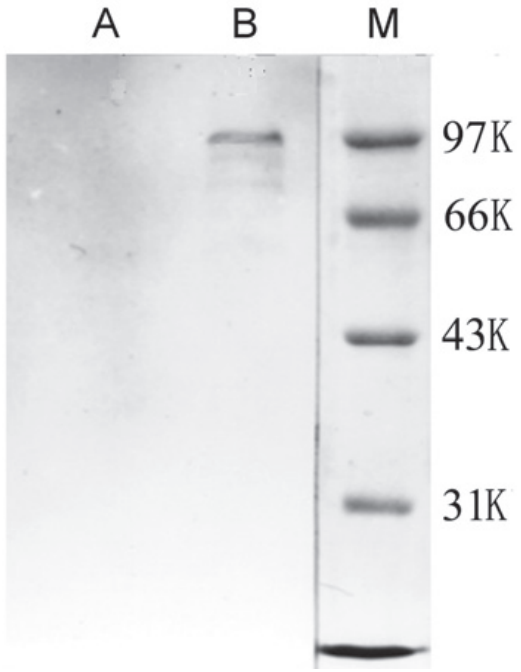


Fig. 1

Western blot analysis of the expressed fusion protein GST-GnN0.7 in *E. coli*

E. coli were transformed with pGEX-4T2 (negative control, lane A) and pGEX-4T2-GnS0.7 (lane B), respectively. Size markers (lane M).

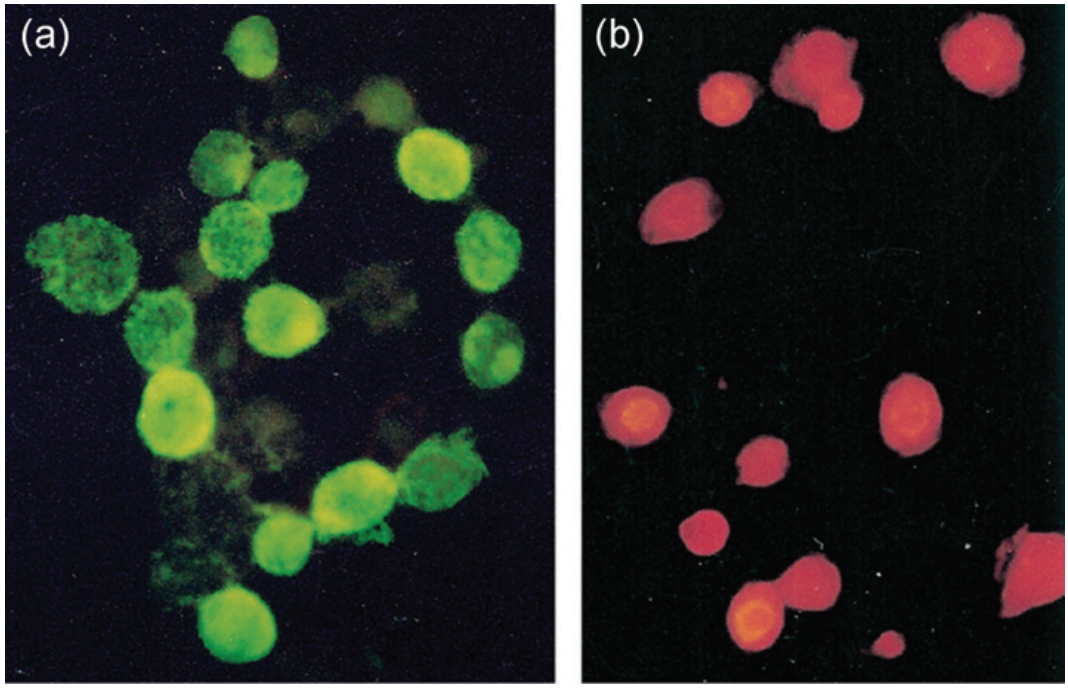
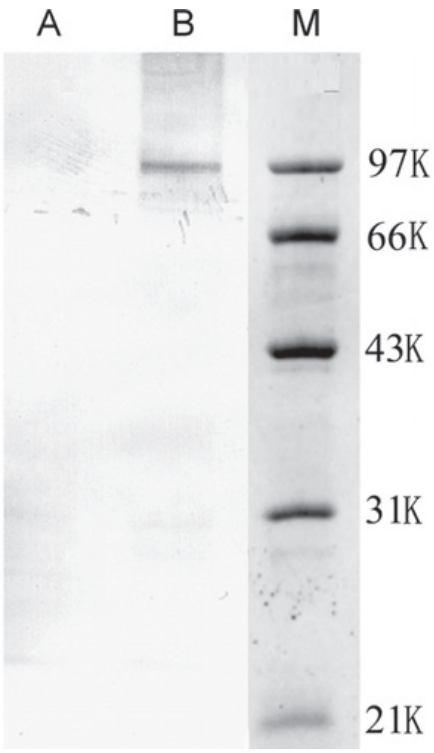


Fig. 2

Immunofluorescence of Sf9 cells transfected for 72 hrs with Bac-GnS0.7 (a)
Sf9 cells infected with AcMNPV as negative control (b).



after the infection. The most intensive fluorescence was observed 72–96 hrs after infection (Fig. 2).

Western blot analysis showed that the expressed fusion protein GnN0.7 was able to bind specifically to HRP-MAb 1A8 and its M_r was about 97 K (Fig. 3).

Immunogenicity of GnN0.7 fusion protein expressed in Sf9 cells

Sera collected from mice immunized with Bac-GnS0.7-infected Sf9 cells and from control mice immunized with AcMNPV-infected Sf9 cells were properly diluted and examined in IFA. The characteristic fluorescence could be observed in the HTNV-infected Vero-E6 cells with the highest dilution of 1:3,200 of mouse sera (Fig. 4).

Fig. 3

Western blot analysis of the expressed fusion protein GnN0.7 in Sf9 cells

Sf9 cells infected with AcMNPV (negative control, lane A) or transfected with Bac-GnS0.7 (lane B), respectively. Size markers (lane M).

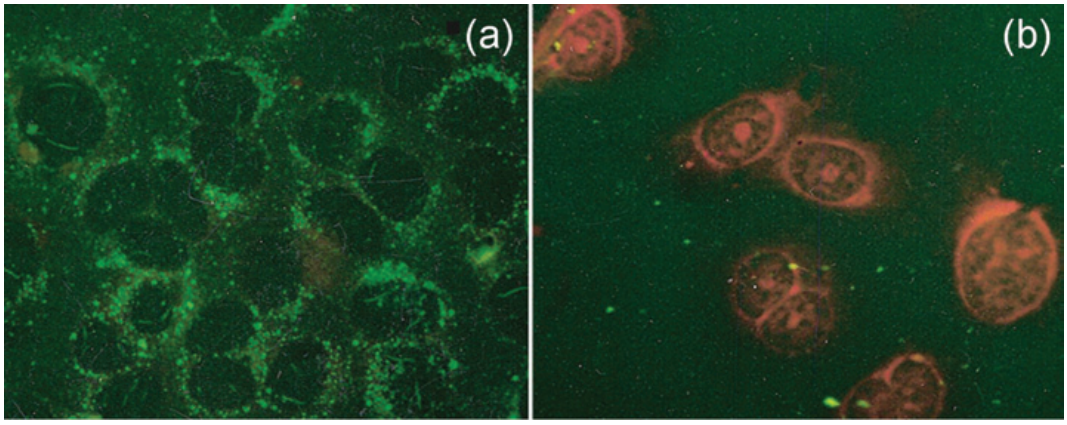


Fig. 4

Immunofluorescence of HTNV-infected VERO-E6 cells with immune mouse sera

Serum diluted 1:3,200 was prepared by immunization with Bac-GnS0.7 (a) or AcMNPV (b) infected Sf9 cells, respectively.

ELISA examination of immune mouse sera showed that the antibodies with specificity to both HTNV N protein and glycoprotein Gn were detected. The ratio (A_{490} positive to negative serum) was more than 6, when sera were tested with N protein and about 3 when tested with glycoprotein Gn. The negative value of A_{490} of all groups were all less than 0.10.

The immune mouse sera were tested also in virus neutralization to determine the ability of the fusion protein GnN0.7 to elicit the production of neutralizing antibodies. We found only low neutralizing antibody titers ranging from 20 to 40, which were detected in 2 of 6 immunized mice.

Cellular immune response was measured by lymphocyte proliferation test (Fig. 5). The proliferation index of splenocytes collected from mice immunized with fusion protein GnN0.7 were higher than the negative control in all mice tested.

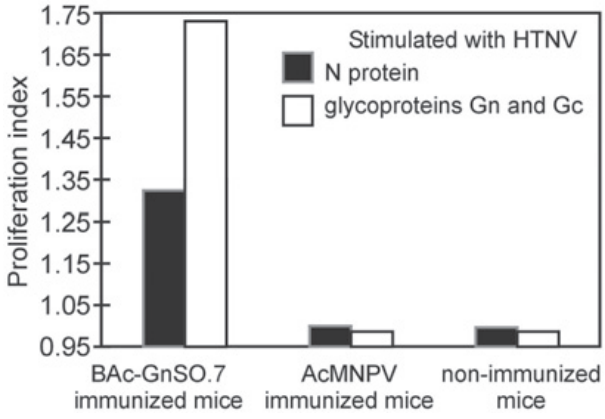


Fig. 5

Effect of stimulation with HTNV N protein and glycoproteins Gn, Gc on the proliferation of immune splenocytes

The splenocytes were collected from mice immunized with Bac-GnS0.7 and AcMNPV infected Sf9 cells or from non-immunized mice.

Discussion

Recently, many scientists focused on the developing of genetically engineered vaccine for HFRS, because of the shortcomings of inactivated classical vaccine (Cao *et al*., 2008). Immunological research on structural proteins of HTNV showed that N protein and both glycoproteins are involved in the eliciting of protective immunity. Among them, the glycoproteins of HTNV may play an important role in evoking neutralizing antibodies for protection against HTNV infection and in the cell-mediated protective immune response (Terajima *et al*., 2002; Pensiero and Hay, 1992). The disadvantage is that the glycoproteins have a relatively low immunogenicity, whereas the N protein of HTNV has the strong immunogenicity and can stimulate production of sera with high titers and long-lasting levels of specific antibodies. Besides, the N protein could also induce cellular immunity against HTNV infection (Lundkvist *et al*., 2002; Park *et al*., 2000; van Epps *et al*., 1999). The previous research in our laboratory proved that the antigenic and possibly some neutralization sites of the N protein were mainly concentrated close to the N-terminus, what corresponded to the 0.7 kb fragment of the S segment (Xu *et al*., 2000, 1992, 1989, 1988). To investigate the possibility of colligating the strongpoint of N protein and glycoprotein, we examined the expression of fused Gn fragment of M segment and 0.7 kb fragment of S segment 5'-end.

Initially, the prokaryotic expression system in *E. coli* was used, since it is cheap and easily managed. Western blot analysis verified that the chimeric gene GnS0.7 could be expressed as an integrated gene. ELISA and Western blot analysis showed that the expressed fused protein signed GST-GnN0.7 was biologically active and had relatively high binding activity against MAb specific to HTNV N protein and a weak binding activity against MAb specific to HTNV glycoprotein Gn. The most rational explanation of this outcome might be the lack of the post-translational modification in prokaryotic cells that affects the correct folding of Gn glycoprotein, what created the different conformation of expressed Gn protein in comparison with the authentic Gn glycoprotein. Moreover, the antigenic sites of glycoprotein Gn are conformational (Zoller *et al*., 1989), therefore Gn expressed in *E. coli* had a low reactivity MAb specific for glycoprotein Gn. However, the low titer of glycoprotein Gn-specific MAb that were used in the test may be another reason.

To obtain the eukaryotic post-translational modification and to improve the biological activity of the expressed proteins, we used a baculovirus-insect cell expression system. In our studies, the HTNV proteins N- and Gn-encoding segments were fused, expressed, and the fused protein GnN0.7 was analyzed. The results proved that the integrated fused protein GnN0.7 was obtained and its antigenic activity was much stronger in comparison with the fused protein GST-GnN0.7 expressed in *E. coli.*

One of our goals was to search for the effective and efficient immunogen suitable for development of genetically engineered vaccine against HFRS. The immunogenicity of the protein GnN0.7 expressed in insect cells was tested by the immunization of mice. IFA titer to HTNV of immune sera was 3,200 and both types of antibodies specifically reacting with N protein and glycoprotein Gn were detected. On the other hand, the titer of neutralizing antibodies was rather low and could only be observed in 2 of 6 immunized mice. Previous study has indicated that there are at least three neutralization sites on HNTV proteins: one on the Gn and two on the Gc glycoprotein (Arikawa *et al*., 1992). Studies on neutralizing MAb escape mutants of HTNV suggested that the neutralization related antigenic sites on the Gn and Gc glycoproteins appeared to be related to each other, both structurally and functionally (Kikuchi *et al*., 1998; Hölling *et al*., 1997; Wang *et al*., 1993). Results of Shi *et al*. (2002) suggested that the Golgi retention signal of HTNV glycoproteins might be dependent on the conformation of oligomerized Gn and Gc complex rather than on the primary amino acid sequence of glycoproteins. Currently, we are studying the expression of glycoprotein Gn only and the influence of such expressed protein on the efficacy and levels of elicited neutralizing antibodies.

Although neutralizing antibodies against HTNV can protect hosts from viral infection, T-cell responses to HTNV are also important in host defense against HTNV. CTL epitopes in the HTNV proteins were identified (Taruishi *et al*., 2007; Maeda *et al*., 2004; Lee *et al*., 2002; Park *et al*., 2000; van Epps *et al*., 1999). In a murine model studied in many reports, CTLs are considered to be important for *in vivo* clearance of HTNV. Adoptive transfer of immune T-cells protected suckling mice from death following infection with HTNV. Also, it was found by transferring T-cell subsets into HTNV-infected nude mice that T-cells expressing CD4–CD8+ markers on their surface were especially important for elimination of infectious virus *in vivo* (Nakamura *et al*., 1985). The lymphocyte proliferation index was also tested and the results showed that the cellular immune response in the mice immunized with Bac-GnS0.7-infected cells was elicited too.

Further studies concerning the antigenic and functional properties of the expressed fused protein and a more detailed research into the expression of HTNV fused N protein and glycoproteins Gn and Gc and their immunogenicity is carried out. These studies should provide a basis for future utilization of fused expressed HTNV proteins as the potential HFRS genetically engineered vaccine.

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