Antibodies against analogous heptad repeat peptide HR212 of Newcastle Disease Virus inhibit virus-cell membrane fusion*

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Abstract Membrane fusion is a key step in enveloped virus entry. Highly conserved heptad repeat regions (HR1 and HR2) of Newcastle disease virus (NDV) fusion protein (F) are critical functional domains for viral membrane fusion. They display different conformations in the membrane fusion states and are viewed as candidate targets for neutralizing antibody responses. We previously reported that an analog of heptad repeat peptides HR2-HR1-HR2(HR212) and HR2 could inhibit NDV induced cell-cell membrane fusion. Here, we show that HR212 can induce the production of highly potent antibody in immunized rabbits, which could recognize full length peptides of both HR1 and HR2, and inhibit NDV hemagglutination and NDV entry. These suggest that either HR212 or its antibody could be an inhibitor of virus-induced cell-cell membrane fusion.

Keywords: NDV, heptad repeat domains, immune response, inhibition of viral entry.

Newcastle disease virus (NDV) is an enveloped virus grouped under the family of *Paramyxoviridae*. Two transmembrane glycoproteins on its envelope, the haemagglutinin neuraminidase (HN) and fusion (F) proteins can elicit a protective immune response[1]. The HN protein initiates the first step in the viral infection, which involves the attachment of the viruses to cellular surface receptors. The attachment is responsible for haemagglutination. The F protein directly mediates a pH-independent virus-cell membrane fusion. Both the HN protein and F protein on the surface of the infected cells can mediate fusion of the infected cells with uninfected cells, causing the formation of syncytia, a prominent cytopathetic effect of paramyxovirus on the cells.

In the fusion process, the F protein undergoes at least three conformational changes to complete the membrane fusion and viral entry: metastable conformation state before fusion, prehairpin intermediate state, and six-helix bundle hairpin state after fusion. In the highly stable six-helix bundle, the HR1 helices form a central trimeric coiled structure that contains three highly conserved hydrophobic grooves that are packed into three HR2 helices in an antiparallel orientation[2]. This structure is coupled viral envelope, targets cell membrane, and promotes viral membrane fusion process, which is a key step for enveloped membrane fusion. In the trimeric prehairpin coiled intermediate, HR1 and HR2 are exposed[3,4]. Studies showed that the prehairpin intermediate structure could keep on for a few minutes[5], thus both the internal N terminal HR1 trimeric coil and the C terminal trimeric HR2 are accessible to inhibitors and are considered as an attractive target for vaccine development[6,7].

We previously proved that HR2 and its analogous HR2-HR1-HR2 (HR212) were potent inhibitors of virus-mediated membrane fusion by mimicking HR peptides interactions in the six-helix bundle and interfering with the subsequent self assembly of the highly conserved endogenous HR domains of the F protein. However, peptides from HR1 are generally far less potent than HR2 for a low tendency to form a trimeric coil[8—10]. Efforts on eliciting high immune response by immunization with free HR peptides or fusion proteins have been proven difficult in success, possibly due to incorrect structural presentation of the peptides[11]. Thus, lowering the conformational flexibility may help in increasing immune responses. Several studies have demonstrated that many immunogenic peptides have a propensity to adopt the well-defined secondary structure in aqueous solution[12,13].

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In this study, we sought to identify whether HR212 antibodies could inhibit NDV entry. HR212 was determined to be more stable than the free HR1 or HR2 peptides based on the formation of a six-helix bundle in solution. The results indicated that the purified HR212 raised a high titer antibody in rabbits. The polyclonal antibody could recognize HR1 and HR2 full length peptides, and could also efficiently inhibit the agglutination of NDV with chicken red blood cells (RBC) and neutralize NDV mediated virus-cell membrane fusion.

1 Material and methods

1.1 Construction of plasmids

The genes encoding HR1 and HR2 segment of NDV F48E9 F protein (Fig. 1(a)) were cleaved from pGEX-6P-1-HR1 and pGEX-6P-1-HR2[9] with restriction enzymes *Bam* HI/*Xho* I and inserted into the similarly digested vector pET-30a (Novagen, Madison, WI, USA). The constructs yielded proteins with N-terminal 6xHis tag, thrombin, and enterkinase cleavage site, and the proteins were named HR1-30a and HR2-30a, respectively. The sequences of the constructs were confirmed by DNA sequencing.

1.2 Protein expression and purification

Expression and purification of HR212, HR1-30a and HR2-30a were performed according to the methods described earlier[8,10].

1.3 Rabbit immunization

For the first immunization, 100 μg of HR212 protein was emulsified in an equal volume of complete Freund's adjuvant (Sigma, USA) and injected subcutaneously into the abdominal region of New Zealand white rabbits. The immunization was boosted three times at a 3-week interval with the same immunogen mixed with incomplete Freund's adjuvant. Pre-immune sera were collected before the immunization. Antisera were collected two weeks after the third boost, and then HR212 specific antibodies were determined by an enzyme-linked immunosorbent assay (ELISA)[14].

1.4 NDV preparation

NDV strain F48E9 was inoculated into 9-day-old embryonic chicken eggs. After 3 days of incubation at 37℃, the allantoic fluid was harvested and clarified by centrifugation (15 min, 12000 *g*, 4℃). HeLa cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. When infection by NDV F48E9 became advanced, the cells were disrupted by freezing and thawing. Cell debris was pelleted by centrifugation (15 min, 12000 *g*, 4℃). Aliquots of clarified supernatants were frozen at 70℃. $TCID_{50}$ of the stock virus was determined by infecting Hela cells with serial dilutions and quantified by cytopathetic formation.

1.5 Haemagglutination (HA) and haemagglutination inhibition (HI) assay

In haemagglutination assay, serial two-fold dilution of NDV virosomes in PBS, beginning at the dilution of 1∶10, was set up in a V-shaped 96-well microtitre plate. An equal volume of 1% chicken RBC was added to each well. The plate was gently shaken to mix the contents and incubated for 15—30 min at room temperature. Haemagglutinating units (HAU) were defined as the lowest dilution able to inhibit the sedimentation of red blood cells. The HI tests were performed by a standard microtest procedure. The HI titre was defined as the reciprocal of the highest serum dilution completely inhibiting agglutination.

1.6 Cell fusion inhibition assay

Sterile 24-well tissue culture plates were seeded with Hela cells in DMEM containing 10% fetal calf serum and grown to confluence overnight at 37°C in 5% CO_2. Serial two-fold dilutions of rabbit antiserum were made in 100 μL of DMEM. 100TCID50 of NDV in 100 μL of DMEM were added to each serum sample. Virus-antiserum mixtures were incubated at 37°C, in 5% CO_2 for 60 min. Confluent monolayers were washed twice with sterile PBS, overlaid with virus-antiserum mixtures (all performances were undertaken in quadruplicate, including control wells containing preimmune sera or mixture of preimmune sera and NDV), and incubated for 72 h. The plates were emptied, and the cells were fixed with 10% formalin, and stained with 0.1% crystal violet (w/v) in PBS. The wells are visualized at low magnification (40x) for presence of multinucleated syncytia. Neutralizing titer was calculated based on Reed-Meunch method.

2 Results

2.1 Proteins were all expressed in soluble form

HR1 and HR2 segments of HR1-30a and HR2-

30a consisted of 136—198 and 449—502 amino acids of F protein respectively; and HR1 and HR2 segments of HR212 consisted of 140—177 and 467—502 amino acids respectively (Fig.1(a)). HR212 (Fig.1 (b)) could be easily expressed in and purified as glutathione S-transferase (GST) fusion protein from *Escherichia coli* in soluble forms at good yields with approximately 7—8 mg fusion protein per liter. Removing the cleaved GST and GST-3C resulted in a highly pure gene product (>95% homogenecity). HR1-30a and HR2-30a were partially soluble at 30℃ and could be easily purified from Ni-chelating sepharose column (Fig.1(c)).

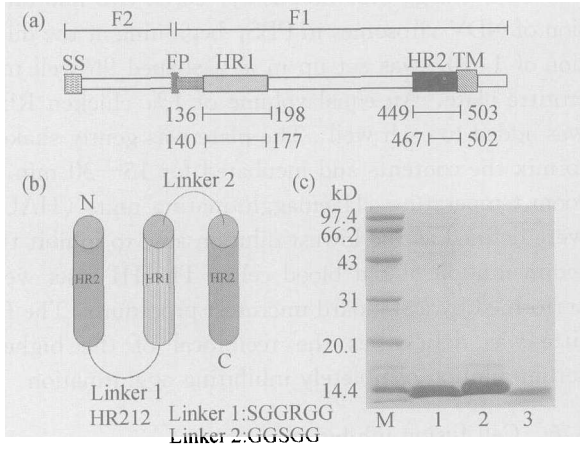

Fig. 1. Schematic diagram of NDV F protein (a) and (b) and SDS-PAGE analysis of the purified proteins (c). In (a) SS, the signal sequence; FP, the putative fusion peptide; The HR1 region, HR2 region and transmembrane segment (TM) are all indicated. (b) is the construct of HR212. In (c) lane 1, HR2-30a; lane 2, HR1-30a; lane 3, HR212; lane M, protein markers.

2.2 The high titer antibody against HR212 could recognize HR1 and HR2 full length peptides

Since HR212 is a protein with alternating HR1 and HR2, the antibody in the rabbit sera might be able to bind to both the HR1 and HR2 full length peptides. As shown in Fig. 2, the antiserum had strong reactivities with HR1, HR2, and HR212. The end-point titers of the sera against HR1, HR2 and HR212 were all 1:256,000 (Fig.2). This suggested that HR212 peptide in a stable state is immunogenic and can elicit high titer antibodies against relevant antigen.

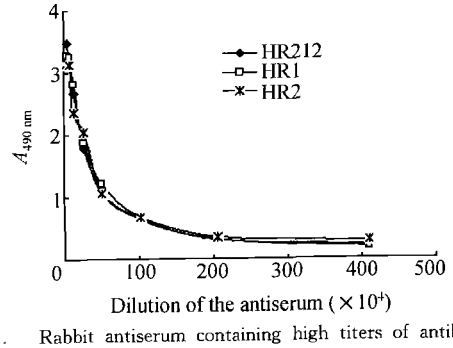

Fig. 2. Rabbit antiserum containing high titers of antibodies. Binding to HR1-30a, HR2-30a and HR212 by rabbit anti-HR212 serum collected 10 days after the third boost at a series of two-fold dilutions.

2.3 Antibody against HR212 could inhibit haemagglutination of NDV

To assess whether the antibody of HR212 in the rabbit serum could block the binding of HN to the sialic acid receptor, we performed HA and HI test. As shown in Fig. 3, the end-point dilution of NDV from allantoic fluid is 1:320, in which a positive pattern is still present. Therefore, the virus titer of the undiluted sample is 320 HA units. HR212 antisera collected two weeks after the third boost had HI activity against NDV. The minimal quantity capable of producing a given HI effect, that is sedimented RBCs in the bottom of the well, is 1:160 dilution of HR212 antisera. The preimmune rabbit sera showed no HI activity. This result indicated that the interaction between HN and its receptor could be significantly inhibited by HR212 antisera.

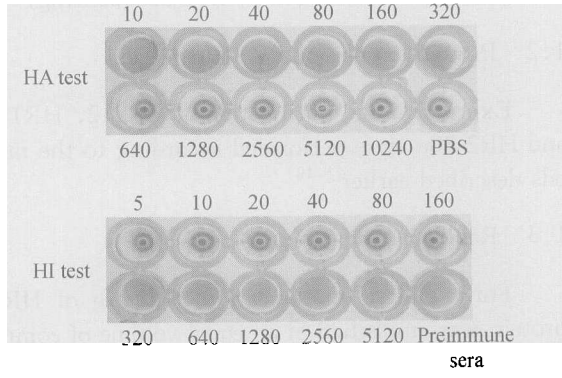

Fig. 3. HA and HI tests. Numbers above or below the wells indicated the dilution folds of the virus or antisera respectively.

Arithmetic HI titer in the range of 2^3—2^5 has been proposed as a good predictor for clinical protection of birds immunized with inactivated vaccine[15]. The arithmetic HI titer of HR212 antisera (160) is far higher than this range. Since HI titer is correlated closely with neutralizing antibody titer, antisera that could block virus-cell interaction may have the potential to counteract infection and protect the cell from virus entry.

2.4 Antibody against HR212 could inhibit NDV induced cell-cell fusion

The mixture of preimmune rabbit sera and NDV

exhibited large sprawling areas of complete fusion in Hela cell (Fig. 4 (a)). Anti-serum showed NDV induced cell-cell fusion inhibition activities with a neutralization titer 1∶11.5. The highest dilution that gave complete membrane fusion inhibition was 1∶4 (Fig. 4 (c)), just like preimmune sera control (Fig. 4 (b)). Moreover, the inhibition effect was also demonstrated in the development of syncytia size and quantity. Hela cell monolayers infected with the mixture of NDV and HR212 antisera at an antisera dilution higher than 1∶8 showed the production of stellate fusion areas interspersed with intact cells and a small reduction of syncytia size and numbers compared with the mixture of NDV and preimmune sera control (Fig. 4 (d)). None of the antisera at a dilution higher than 1∶32 possessed such fusion inhibitory effect against NDV. They showed the same as the mixture of NDV and preimmune sera control (Fig. 4(a)).

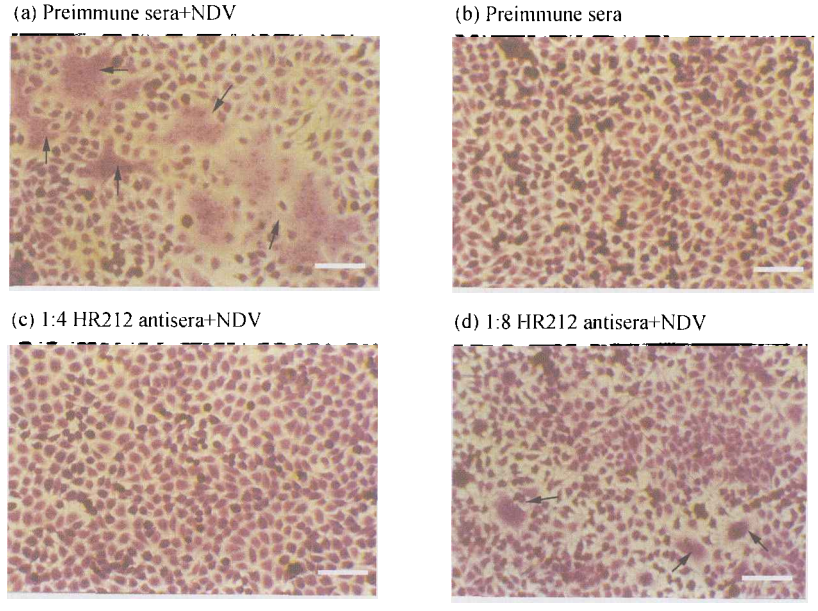

Fig. 4. NDV fusion inhibition by the antisera against HR212 (bar = 30 μm). Arrows in (a) and (d) indicate syncytia.

These results indicated that the antibody against HR212 might be able to effectively inhibit syncytium formation in a concentration-dependent fashion when reacting with the antigenic structures that appeared in the prehairpin intermediate of F protein.

3 Discussion

In the current fusion model of paramyxovirus, fusion is triggered by HN-receptor interaction[1]. One way of antisera membrane fusion inhibition may be through interfering with HN-receptor interaction and precluding the subsequent steps of fusion and viral entry. The high HI activity of HR212 antisera might be due to a simple steric effect of the large mass of IgG complete structure. According to this view, IgG has been shown to inhibit directly the attachment of radiolabelled virus to RBCs[16,17].

The other explanation is that the high HI activity of HR212 antisera relates to HN-F specific functional interaction. It has been suggested that fusion involves a type specific interaction between the HN and F proteins, and their cofunction is necessary for syncytium formation, a characteristic of paramyxovirus membrane fusion[18]. Now, the HN-F interaction occurs whether before or after HN attachment is debated. Two models have been proposed. One model proposes that HN and F proteins interact only after HN attachment and this interaction initiates F protein conformational changes required for fusion[19,20]. An alternative model is that HN and F proteins form a metastable complex prior to HN attachment. HN attachment with a concomitant conformational change promotes F protein a cascade of conformational changes resulting in the formation of a six-helix bundle structure composed of HR1 and HR2[18,21]. Both models share the common idea that HN protein undergoes conformational changes upon attachment. Supposing that this specific HN-F interaction occurs before attachment, whether there is an interaction inducing conformational changes of HN and F proteins and promoting HN attachment is not known yet. The specific interaction of HN-F complex may be through HN stalk region and HR2 of F protein in the prefu-

sion state[22,23]. In this study, the high HI activity of HR212 antisera could be explained by the second model. The HR212 antibody bound to HR2 of F protein in the prefusion state might interfere with the HN-F complex formation and its concomitant conformatioal topographical rearrangements, which may be necessary for receptor binding, resulting in a HN protein conformation at the receptor binding site, thus giving a high HI titer.

Neutralization is an *in vitro* process in which virus binds antibody and loses infectivity, and it correlates strongly, although not exclusively, with protection from infection *in vivo*[24]. The neutralizing activity of HR212 antisera is probably due to HR212 antibody binding to the HR1 and HR2 domains with high affinity in the fusion intermediate, blocking the conformational change that results in a six-helix bundle and hence preventing the fusion of NDV with the host cells. We found that the titers of neutralizing antibodies generated by our designed fusion proteins were low. Similarly, monoclonal antibodies specific for the six-helix bundle have been found to be non-neutralizing[25,26]. Maybe large amounts of antibody are required for HR212 antisera to elicit effective neutralization, because the neutralizing activity of it displayed a concentration dependence. This might be due to the insufficient functional affinities and steric hindrance of the large mass of IgG limiting the neutralizing efficacy of the antibody[24,27,28]. The low level of exposure of HR2 epitope, due to its location close to the viral membrane, may also explain the low neutralizing efficiency of anti-HR212 antibody. Consistent with these analyses, a study demonstrated that it took between 10 and 100 μg/mL 2F5, depending on the primary isolate, to neutralize HIV[14,29], which suggests that the monoclonal antibody has a low affinity for its epitope and thus the need for high concentrations is necessary to achieve effective neutralization.

In conclusion, HR212 elicited high titer antibodies recognizing HR1 and HR2 domains in rabbit. The antibodies had HI activity and neutralizing activity. The blockage of HR212 antisera to the binding of HN to sialic acid receptor and the transition from prehairpin intermediate state to the postfusion state is the possible mechanism of action of anti-HR212 antibody in NDV infection.

Recently, antibodies raised against covalent trimers of HIV HR1 trimeric coil N35CCG-N13 showed highly potent inhibition in HIV-1 fusion, which means that the prehairpin intermediate state is accessible to antibodies and that accessibility is not restricted by either antibody size or the presence of a kinetic barrier[30]. Therefore, adding more HR1 to HR analogue may elicit high titer antibodies and the resultant antisera might be more effective in neutralizing virus infection.

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