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Antigenic Characterization of the Glycosylated E2 Proteins of Classical Swine Fever Virus

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E2 protein (E2) is one of the major structural proteins of the Classical Swine Fever Virus (CSFV). It is the major protective antigen of CSFV. The N-glycosylated E2 protein (Gly-E2) which had been glycosylated in vitro will enhance the immune response in theory. Therefore, in order to evaluate the immune efficacy of Gly-E2 of classical swine fever virus, the Gly-E20dendritic cells (DCs) and T cells were co-cultured and the BALB/c mice were inoculated with the Gly-E2 after that the E2 was glycosylated successfully. Sequentially, the IL-40IFN-γ and IgG levels in cell supernatant and in serum of BALB/c mice were measured by ELISA technology. The results showed that the glycosylation of E2 enhanced antigen presentation of dendritic cells significantly, and promoted the Th1 and Th2 immune response at the same time. Furthermore, the Th2 humoral immune response was more significant. The Gly-E2 excited the body to produce faster and more durable antibody protection and a better immune memory than that of CSFV and E2. Consequently, the results proved that the Gly-E2 possess a good preventive effect to classical swine fever. Therefore, the Gly-E2 will have a very deep development potential for the exploitation of new vaccines as a preferable antigen.

Key words: Classical swine fever virus, E2 protein, Glycosylation, Immune response.

Classical swine fever (CSF) is a highly infectious and contagious disease of swine, causing morbidity and mortality among swine worldwide[12]. Classified by the Office International des Epizooties (OIE) as category A, The outbreaks of CSF usually cause huge economic losses in the pig industry[14,21]. Clinical symptoms of the disease are high fever, hemorrhage, necrosis and infarction of some organs[15, 22].

The two existing strategies to control CSF epidemic are systematic prophylactic vaccination with live attenuated vaccines (such as C-strain) and non-vaccination stamping-out policy[2]. Compared to the high costs and in some cases inadequacy of stamping out, live attenuated vaccines (LAV) are inexpensive and can induce complete protection against virulent CSFV, so LAV are still widely used in the CSF endemic areas[17, 26]. Eradication of CSF based on the LAV vaccination alone is difficult because differentiation between infected and vaccinated animals (DIVA) is impossible based on the antibodies induced[1]. Marker vaccine and companion serological diagnostic test is thought to be a promising strategy for future control and eradication of CSF[5]. Recently, E2 subunit vaccines produced using the baculovirus protein expression system and yeast expression system were shown to induce serum antibodies with high virus neutralizing titers and to provide good protection against CSFV[14,19]. The disadvantage of these vaccines is that more expensive than modified live vaccines. The new marker vaccine, which is cheap and effective, becomes the focus of research.

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DCs is the most powerful professional antigen presenting cells that can activate naive T cells[4]. Mannose receptor (MR) is endemic membrane receptor of DCs, which can identify a variety of sugar molecules such as mannose, fucose and N-acetylglucosamine etc on the cell surface or in the cell walls of pathogens. Previous results showed that the immune effect of glycosylated E2 protein was better than of E2 protein on rabbit model[27], and can effectively enhance the humoral immune response and the optimum dosage was 50 µg on the mice model[6,25].Therefore, the glycosylated E2 protein possess a very deep development potential of new vaccines as the preferred antigen. The project studied immune response of the E2 protein glycosylation in vitro and vivo, which would lay the foundation for the new marker vaccine of CSF.

MATERIALS AND METHODS

Expression and purification of E2 protein

The plasmids pET 32a-zE2 were transformed into the *E. coli* strain BL21 (DE3)/pLyS and cultured in Lysogeny broth (LB, sigma, USA) containing 50 mg/mL ampicillin (the ratio was 1:100), an zE2 gene has been sequenced by company (Fig.1) . Cells were induced with 0.4mM of isopropyl-b-Dthiogalactopyranoside (IPTG) for 4h at 37°C, 225 rpm. The cells were harvested by centrifugation at 1,500×*g* for 20 min at 4°C and re-suspended in apropriate amount of PBS (pH7.4) buffer, and sonicated. The protein was furthr purified with Ni^{2+} NTA-agarose and DEAE-Sepharose FastFlow chromatography (Novagen). Sequentially, the purified E2 protein were analyzed by SDS-PAGE and Western blotting.

Glycosylation of E2

The µ-diaminocaproic acid glycosylation sites at the N-terminus of E2 were glycosylated by 4-Aminophenyl α-D-mannopyranoside using methods previously described[3]. Briefly, 2 mg of E2 protein were solved into 200µL of DMSO, and the 4 mg of α-D-mannopyranoside and 2µl of N-methylmorpholine were added into the solution. The reaction mixture was stirred overnight at 150 rpm and then added 100µL of 1 M Tris-HCl pH9.5 into the mixture for another 1h. The Gly-E2 protein were lyophilized and stored at 20° C.

Immune response in BALB/c mice after inoculation with glycosylated E2

Eighty-four BALB/c mice(6 weeks old) were randomly divided into four groups. Three groups were intramuscularly immunized with Gly-E2, E2 and vaccine, respectively. A control group was inoculated with PBS. At 7, 14, 21, 28, 35, 42 and 56 days postinoculation (dpi), three mice from each group were sacrificed and serum was harvest respectively, and levels of serum IgG, IFN-γ, and IL-4 were measured with the commercial with ELISA kits (R&D Systems, USA).

Generation of DCs and T cells

Peripheral blood mononuclear cells (PBMC) were obtained from Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation from BALB/c mice and cultured in RPMI-1640 containing 10% fetal bovine serum, 20ng/ml recombinant murine granulocyte macrophage-colony stimulating (GM-CSF), and 20ng/ml recombinant murine interleukin(IL)-4 (both 20ng/ml) for 7 days. The cells were harvested and analyzed by flow cytometry .

The superficical cervical lymph nodes were harvested from the BALB/c mice (6 weeks of age) and cut into small pieces under sterile conditions, and then passed through a 300 mesh sieve(sigma, USA) to remove tissue fragments. The lymph node single-cell suspensions were prepared with lymphocyte separation liquid (sigma, USA) and cultured for 1h (37°C , 5% CO_2). Finally, the T cells were isolated by AET-E rosette sedimentation method[10].

The immune response in vitro

In order to evaluate the efficiency of cellular responses induced by Gly-E2 in vivo, the DCs, 5×10^3 cells per well in 96-well cell culture plates, were pre-incubated with 0.03 nmol/L Gly-E2, E2 and CSFV, respectively, and were incubated for 24 h at 37°C. A blank consisting of only DCs with PBS was used as a negative control. The T cells were then added to the 96-well plates, 5×104cell/well. Sequentially, the cells co-culture supernatants were obtained in 24, 48, 72 and 96 h and the levels of IFN-γ and IL-4 were measured using the commercial ELISA kits (R&D Systems, USA).

Data analysis

All experiments were performed

independently at least 3 times and the data were analyzed as the mean ± SD. Statistical analysis was performed by paired Student's *t* test ($p < 0.05$ was considered significant).

RESULTS

Expression, characterization and glycosylation of E2 protein

An approximately 34KD protein were successfully purified from lysates of transformants by means of sequential Ni-NTA agarose chromatography and ion exchange chromatography based on the result of Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2A). The purified protein could be recognized by western blotting analysis using the rabbit anti-CSFV polyclonal antibodies (Fig. 2B). The lysine μ- amino at the N-terminus of E2 protein were successfully glycosylated with 4-Aminophenyl α-D-mannopyranoside, and characterized by mass spectrographic analysis and SDS-PAGE (Data not shown).

The immune response to Gly-E2 in vivo

Levels of the IgG antibody rose to the highest level on 28d after E2 and Gly-E2 inoculation, which were faster than that of CSFV group (increasing to the highest level on 35d). Furthermore, the IgG levels of the Gly-E2 group were significantly higher than the other three groups ($P<0.05$)during the phases of immune response. For immunizing 56 days after Gly-E2 inoculation, these results indicated that the Gly-E2 can stimulate long-lasting humoral immune response quickly and lastingly (Fig. 3-A).

The levels of IL-4 were determined by an ELISA assay using serum samples separated from the whole blood . The results showed that the IL-4 levels were increased from 7 days to 35days, and IL-4 levels were gradually decreased and achieved to a stable level for 42days after inoculation. But the levels in the groups inoculated with CSFV0E2 and Gly-E2 were markedly higher than that in controls(PBS group) ($P<0.01$). At the time of 7, 21 and 42d days after inoculation, the levels of Gly-E2 group were not notably different from CSFV

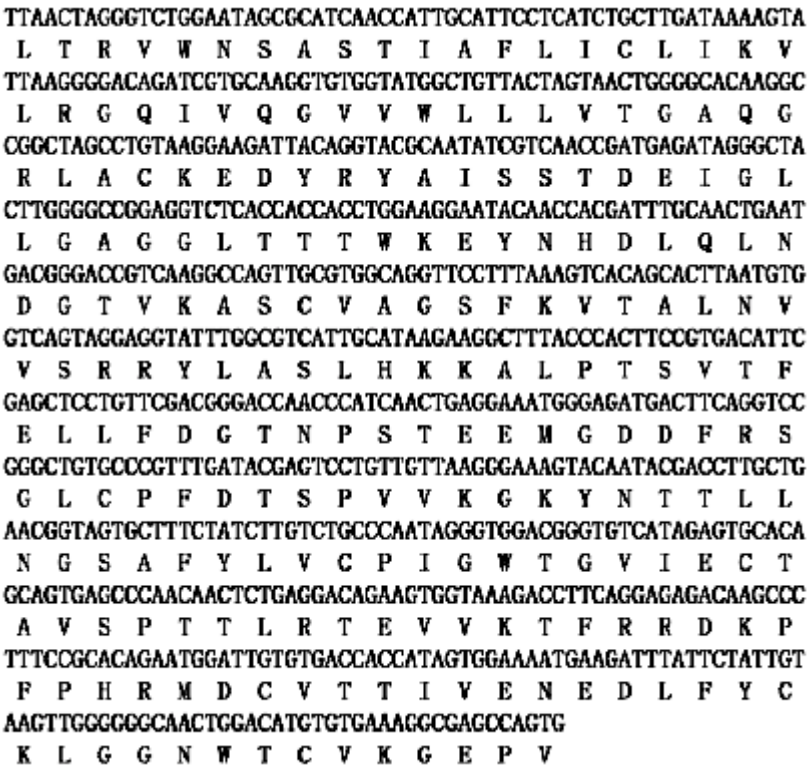

Fig. 1. The nucleotide and deduced amino acid sequences of zE2 gene

and E2 groups. However, at the time of 14, 28, 35 and 56 days after the Gly-E2 inoculation, levels of IL-4 were remarkably higher than the other three groups, that demonstrated that the Gly-E2 can induce faster immune response and remain at high levels for a longer period of time (Fig. 3-B).

The levels of INF-γ in serum were evaluated respectively at the same time. The results indicated that the levels of INF-γ were increased from 7 to 28 days after inoculation with CSFV, Gly-E2 and E2, and INF-γ levels begun to decrease from the 35th day. The levels of INF-³ in CSFV group increased faster and higher than that of E2 and Gly-E2 groups on the seven day after immunization (P>0.05). However, the levels of INF-γ in the three groups were not considerably different on the 14th day. INF-γ levels were remarkably higher on days 21 and 28 compared the Gly-E2 group with E2 group (P<0.01), but not significantly different with that in CSFV group (P>0.05). Although the INF-γ levels in the Gly-E2 group were decreased on the 35th days after vaccination, they still were significantly higher than that in the other two groups for 42 days (P<0.01). To 56 days, the levels of INF-γ became not extraordinarily different between Gly-E2 group and CSFV group (P>0.05, but still significantly higher than that in E2 group (P<0.01) (Fig. 3-C).

The immune response to Gly-E2 in vitro

To evaluate the immune response induced by the Gly-E2 in vitro, the T cells were put into the co-culture system after that the immature DCs were

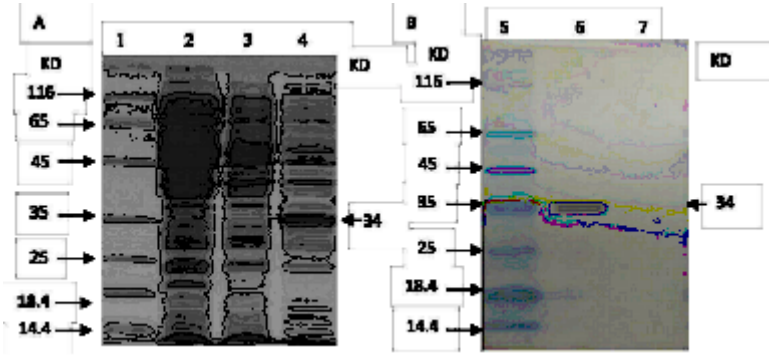

Fig. 2. The expression and identification of E2

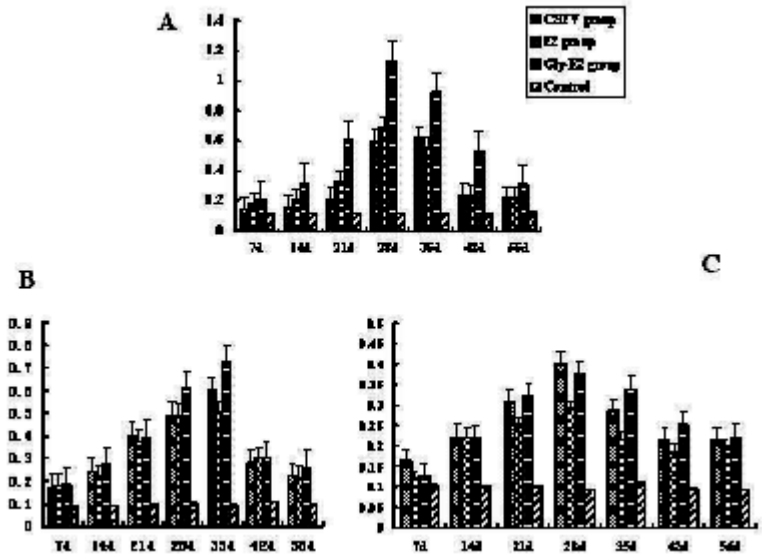

Fig. 3. The immune response to Gly-E2 in vivo

co-cultured with the E2, Gly-E2 and CSFV for 24h at 37°C and 5% CO_2, respectively. The supernatant levels of IL-4 and INF-γ were analyzed after 4, 8, 12, 24, 36, 48 and 72 h of co-culture using ELISA assay. The results showed that the Gly-E2 induced immune response more effectively than the E2 and CSFV in vitro. Moreover, the immune response induced by the Gly-E2 was mainly the Th2 immune response, the level of Th1 immune response induced by Gly-E2 was also higher than those in the other two groups.

The co-culture systems were co-incubated for 4h, the levels of IL-4 in Gly-E2 group and E2 group were professionally higher than that of CSFV group (P<0.01). The levels of IL-4 were similar in the three groups after 8h of culturing (P>0.05). The levels of IL-4 in the Gly-E2 group and CSFV group were significantly higher than that of E2 group within 12-24h, (P<0.01). In addition, the level of IL-4 in the Gly-E2 group increased the highest level on at 24h after culturing, and it was extremely higher than that of the other two groups (P<0.01). The co-culture systems were cultured during 36 to 72h, the levels of IL-4 for all three groups decreased, however the level of IL-4 in the Gly-E2 group was still higher than that of the other two groups (P<0.01) (Fig.4-A)

The co-culture systems were cultured for 4-8h, the levels of INF-γ in the Gly-E2 group and the E2 group were remarkably higher than that of the CSFV group (P<0.05). The levels of IFN-γ between the CSFV group and E2 group were not significant, but they were efficiently lower than that of Gly-E2 group at 12h post-culture (P<0.01). At 24h, the IFN-γ levels of all three groups rose to highest level, also, the IFN-γ level in the Gly-E2 group was notably higher than the other two groups. The levels of IFN-γ of all three groups begun to decrease and were not different significantly from 36h onwards (P>0.05), and the level of IFN-γ in the Gly-E2 group was still higher than the other two groups at 48-72h (P<0.05) (Fig.4-**B)**.

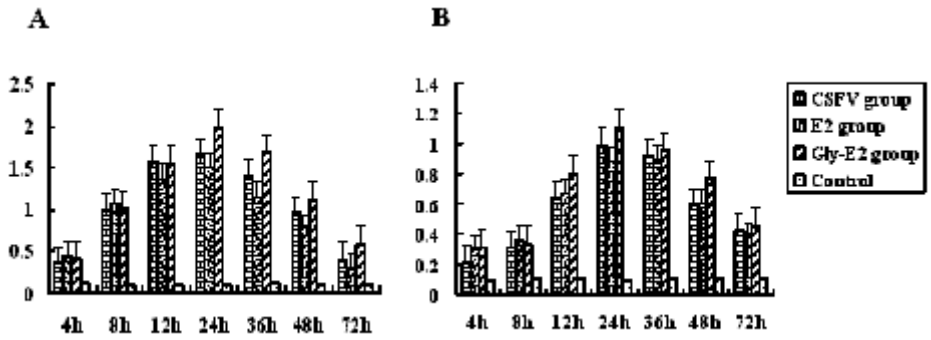

Fig. 4. The immune response to Gly-E2 in vitro

DISCUSSION

Vaccines against CSFV present many new scene with the rapid development of biotechnology[12,21]. Marker vaccine is to reconstruct CSFV that is based on the absence of one or more antigen or antigenic determinant, but its immunogenicity keeps unchanged, which is no substitute for sanitary measures[26]. Therefore, the pigs inoculated with vaccines and infection of pigs with CSFV can be distinguished by the methods for detection of antibodies.

DNA vaccine is one of the important emerging vaccine technology in recent years. The vaccine has been widely used to develop CSFV vaccine, which can induce humoral and cellular immune response[18]. But the animals need to be inoculated many times, which can provide good protection against CSFV. Accordingly, it is the key issue for enhancing the immunogenicity of DNA vaccine. The genetic recombination vaccines using pox virus or pseudorabies virus as vector, to express the E2 protein, can provide protections against two kinds of virus, but its clinical safety

and efficacy are still controversial question. Therefore, novel concepts of vaccines against CSFV is focused on the development of the safe, efficient and stable vaccine.

Structural components of CSFV include the capsid (C) protein and glycoproteins: E^{rns} , E1 protein and E2. E2 is the most immunogenic and transmembrane protein of the CSFV glycoproteins that can efficiently induce neutralizing antibodies against CSFV[13,19,23]. In the study, the results also demonstrated that the E2 possessed high immunogenicity and induced strong Th2 immune responses both in vivo and in vitro settings. Furthermore, the IgG levels of the Gly-E2 group rose to the highest level on 28d, which was significantly faster and higher than that of the CSFV group (the IgG levels reached a maximum on 35d) ($P < 0.05$).

The glycosylation, the attachment of sugar moieties to proteins, is an important post-translational modification of protein, which is the non-enzymatic reaction between the protein and glucose (aldehyde sugar). The sugar chains on the surface of the protein can have a profound effects on the structural properties of protein[23,24]. E2 glycoprotein contains five potential glycosylation sites, and each site can connect with a 2 Ku to 3 Ku oligosaccharides. In this research, the E2 in the CSFV strain contains three É - lysine glycosylation sites using glycosylation sites prediction software[4].

The surface of immature DCs express a large number of MR and use it for efficient capture of the glycoprotein antigens , the MR can deliver a large number of ligands to recycle in the endocytosis system when the mannosylated antigens were ingested by regulation of MR[8]. Furthermore, the internalized antigens can combine with the MHC class II molecules and be submitted to the cell surface[4,7,20]. The mannosylated antigen can fast recycle between cell membrane and endosome so that the antigen internalized by phagocytic cells within 15 min can induce cell response efficiently. On the other hand, the CSFV infection can induce the IFN-α production[9]. Therefore, based on the MR in the DCs membrane surface, the Gly-E2 could theoretically enhance DCs the ability of antigen uptake, presentation and immune response by MR mediated. At present the highly glycosylated analogues of human erythropoietin has been successfully expressed in yeast by glycosylation engineering, so that the half-life of the drug prolonged by 3 times in rats and dogs[16]. In animal husbandry, the N- terminal of CSFV E2 was firstly glycosylated in vitro and the immune effects of Gly-E2 were evaluated in vitro and vivo. Compared with E2 and CSFV, the Gly-E2 enhanced Th2 humoral immune response and created a good immune memory. Therefore, the Gly-E2 should be spread and applied as the preferable antigen of vaccine. The application of Gly-E2 must be able to provide a novel concept for the development of the prevention and control method of CSFV.

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