A Fusion Protein of IgG Fc and Mouse-Derived Antigen on the Surface of Pseudorabies Virus Particles Does Not Accelerate Production of Harmful Auto-Reactive Antibodies

Haruko OTA1, Yasuhiro TAKASHIMA2, Yoshihiro HAYASHI1 and Yasunobu MATSUMOTO1)*

1)Department of Global Agricultural Science, Graduate School of Agricultural and Life Sciences, the University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657 and 2)Department of Veterinary Parasitological Diseases, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagito, Gifu 501-1193, Japan

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ABSTRACT. Previously we reported that immunization with pseudorabies virus (PRV), harboring chimeric Fc on the surface of the virus particles (PRV/Fc), induced higher immune responses than normal PRV particles. The chimeric Fc was fused with mouse transferrin receptor of transmembrane domain (mTR) and the Fc region of immunoglobulin G1. Since it has been reported that some chimeric protein of Fc and self-antigen induce auto-reactive antibodies, in this present study, we examined whether PRV/Fc induces auto-reactive antibodies that react with mTR. PRV/Fc immunized mice produced higher levels of anti-PRV antibodies and antibodies that reacted with mouse-derived 3T3/A31 cells (A31 cell), compared to normal PRV immunized mice. However, antibodies that reacted with mTR in A31 cells were not detected in both Western blot analyses and indirect immunofluorescence assay. The antibodies reacted with an antigen of approximately 16 kDa in A31 cells, but this antigen has a different molecular mass from that of mTR. The antibody also reacted with the antigen of approximately 16 kDa in RK13 cells in which the virus had been propagated. In addition, antibodies induced by immunization with normal PRV also reacted with the same antigen in A31 and RK13 cells. Moreover, neither kidney disorders, in which high levels of mTR were expressed, nor clinical symptoms of autoimmune diseases were observed in mice immunized with either PRV or PRV/Fc. These results indicated that the antibodies were not induced by mTR-Fc, but were instead induced by trace amounts of RK13 derived antigens contained in PRV or PRV/Fc preparations, and cross-reacted with equivalent molecules in mouse derived A31 cells. Therefore, this study confirmed that immunization with PRV/Fc did not induce harmful auto-reactive antibodies.

KEY WORDS: chimeric IgG1 Fc, Fc, PRV.


Pseudorabies virus (PRV), a member of the herpesviridae alphaherpes subfamily, is the etiological agent of Aujeszky’s disease, which is often fatal in newborn pigs. However, adult pigs can be infected subclinically [3], and latent infection in adult pigs can be reservoirs for PRV. To protect pigs from Aujeszky’s disease, live attenuated and inactivated vaccines are currently available. However, the vaccination can produce clinically infected virus reservoir pigs without any clinical symptoms, since the current vaccines are unable to prevent infection from PRV. Therefore, in order to control and eradicate the development of a more effective vaccine that can protect pigs from virus infection would be valuable.

Recently, we established a new strategy to improve the vaccine for Aujeszkey’s disease using PRV particles harboring Fc domain of IgG [7]. It is known that the antigen-antibody complex induces a much greater level of immune reactions. This is due to an increased uptake of the antigen-antibody complex into antigen presenting cells (APC), through the Fc receptors (FcRs) on the APC surface, resulting in increased processing and presentation to the immune system [9, 10]. It was also reported that immunization, with antigen fused with the Fc portion of the IgG molecule, had enhanced antigen-specific CD4+, CD8+ and B cells, compared with immunization using a normal antigen [11]. Therefore, immunization with a complex of virus antigen and Fc has the potential to be an improved vaccine. Host cells, which stably express chimeric fusion proteins containing Fc molecules on the cell surface, have been constructed

Fig. 1. Establishment of PRV particle harboring Fc of IgG. Genes that encode the Fc region of mouse IgG1 and mTR were ligated and transfected into RK13 cells. Fc domain was expressed on the surface of RK/SK cells as a fusion protein with mTR. The Fc was incorporated into the virus particle (PRV) after the PRV invaded the RK/SK cells and replicated, resulting in the conversion of PRV/Fc.
using our established vaccine procedure, as shown in Fig. 1 [7]. The PRV infecting the modified host cell incorporates the fusion protein, containing the Fc domain from the host cell surface, into the virus particle when the progeny viruses egress from the host cells (Fig. 1). Therefore, the resulting progeny viruses have Fc molecules on their virus particle surfaces, without any modification of the viral genome. Immunization with the Fc-harboring virus particle induced a PRV-specific immune reaction more effectively than immunization with the normal virus [7]. These results indicated that the Fc harboring virus has great potential as a new vaccine strategy.

It is known that fusion proteins of self-antigen and Fc domain can induce auto-reactive antibodies. Auto-reactive antibodies specific for self CD20 antigen on B cells were induced by immunization with a CD20 peptide fused with human IgG Fc [2]. It was also reported that immunization with the chimeric fusion proteins human IgG4 Fc, and mouse Igα or Igβ produced specific auto-reactive immune responses against Igα and Igβ, respectively [5, 6]. The fusion protein, which was used in our newly established vaccine strategy [7], contains the trans-membrane region of mouse transferrin receptor molecule on its N-terminal and the Fc domain of mouse IgG1 on its C-terminal (Fig. 1). The fusion protein on the PRV particle could also induce auto-reactive antibody specific to mouse derived TR antigen. Autoimmune reactions and/or over-immune reactions sometimes mediate several diseases [4, 8].

In this study, to confirm the safety of our newly established vaccine strategy from autoimmune diseases, we analysed whether the Fc harboring PRV particles induce auto-reactive antibody and/or any diseases caused by the autoantibodies.

**MATERIALS AND METHODS**

**Cells and viruses:** CPK derived from porcine kidney cells, RK13 derived from rabbit kidney cells, and cell lines expressing Fc domain of mouse IgG1 [7], were cultured in Eagle’s minimum essential medium (E-MEM, Nissui, Tokyo), supplemented with 7.5% fetal calf serum (FCS) and 1 µg/ml of kanamycin. PRV Bucharest strain (BUK) was prepared in CPK, RK13 and RK/SK established cell lines.

**Mice:** Eight-week-old female BALB/c mice were purchased from Japan CLEA (Tokyo, Japan). Animal experiments were carried out under the control of the Animal Research Committee in accordance with the Guidelines on Animal Experiments at the University of Tokyo.

**Virus enrichment and inactivation for immunization:** CPK, RK13 and RK/SK cells were infected with PRV (BUK) at a multiplicity of infection of 1.0. The supernatants of infected cell cultures were harvested 24 hr post infection, and centrifuged at 3,000 rpm for 5 min to remove cell debris. The supernatants were then centrifuged at 27,000 rpm for 2 hr to pellet the virus virion. The pelleted virions were suspended in PBS at a concentration of 1 × 10^6 pfu/ml. The enriched virus was inactivated by treatment with 0.1% formaldehyde. It was confirmed that the formaldehyde treated virus did not form any plaque on a monolayer of CPK cells. The inactivated viruses were used as antigens for immunization.

**Immunization and challenge:** Five or six female BALB/c mice were immunized for each treatment. The enriched and inactivated viruses, at the titer of 5 × 10^6 PFU (before inactivation), were suspended in 50 µl of PBS and injected into the left footpad of each mouse. Blood samples were collected 3, 8 and 26 weeks post immunization. Twenty six weeks after immunization, 20 LD_{50} (approximately 3,200 PFU) of PRV (BUK) was injected intraperitoneally.

**ELISA:** Each well of 96-well ELISA plates was coated with antigens as follows: For anti-PRV antibody detection, enriched PRV was prepared in RK13 cells, diluted in a carbonate buffer (74 mM NaHCO₃, 26 mM Na₂CO₃, pH 9.6) at a concentration of 5 × 10^6 pfu/ml, and 100 µl of the diluted virus was added into each well. For auto-reactive antibodies detection, 3T3/A31 (A31) cells derived from mouse embryos were lysed and then diluted in a carbonate buffer at a concentration of 1 g/ml. After incubation at 4°C overnight, the plates were washed with PBS, and 100 µl of 3% skimmed milk was added to each well. After incubation for 40 min at room temperature, 1:100 diluted serum samples, harvested from immunized mice, were added and incubated at 37°C for 40 min. The antibodies were washed with PBS and then 1:3,000 diluted horseradish peroxidase-conjugated rabbit anti-mouse IgG, IgG1 or IgG2a antibody (ZYMED, San Francisco, CA) was added. After incubation for 40 min, substrate o-phenilendiamine and H₂O₂ were added, and after 30 min the plate was read at OD 490.

**Histopathology:** Kidneys were collected from the mice 26 weeks after immunization, fixed in a formalin solution and embedded in paraffin. Sections were stained with hematoxylin and eosin.

**Western blot analyses:** A31 cells and RK13 cells were collected and suspended with the sample buffer (0.05% bromophenol blue, 10% glycerol, 2.0% SDS, 0.05 M Tris-Cl, 6.0% 2-mercaptoethanol, pH 6.8), incubated at 100°C for 5 min and subjected to electrophoresis in SDS-polyacrylamid gel. The separated proteins were transferred to Immobilon-P Transfer Membrane (Millipore), and the membrane was blocked with 3% skimmed milk for 1 hr at room temperature. The membranes were incubated with sera from immunized mice at a dilution of 1:500 for 1 hr at 37°C. After three washes in PBS, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (ZYMED) at a dilution of 1:3,000 for 1 hr at 37°C. After three washes with PBS, the membranes were developed for peroxidase activity with ECL Western Blotting Detection Reagents (Amersham Biosciences). The fluorescence was detected by exposure to X-ray film (Kodak X-Omat).

**Indirect immunofluorescence assay:** A31 cells were collected and exposed with 1:100 diluted serum samples harvested from immunized mice or rat anti-mouse TR antibody (BioLegend, San Diego, CA) at room temperature for 1 hr. After washing with PBS, cells were incubated with 1:100...
diluted FITC-conjugated rat anti-mouse IgG (H+L) antibody (eBioscience, San Diego, CA) or 1:320 diluted FITC-conjugated anti-rat IgG (Sigma, Saint Louis, MO). After incubation for 1 hr at room temperature, the antibodies were washed with PBS, mounted in ProLong Gold antifade reagent (invitrogen) and examined with a fluorescence microscope.

RESULTS

Antibody production by immunization with PRV harboring Fc: To examine the antibody production after immunization with PRV particles harboring Fc (PRV/Fc), mice were immunized with $4 \times 10^6$ pfu of PRV or PRV/Fc. Consistent with our previous report [7], at 3 weeks post immunization higher levels of PRV-specific IgG, IgG1 and IgG2a were detected in mice immunized with PRV/Fc, compared to mice immunized with normal PRV (Fig. 2). In addition, the levels of PRV-specific IgG production, including both IgG1 and IgG2a in PRV/Fc immunized mice, still remained high 26 wks post infection (Fig. 2). These results suggest that the enhanced ability of PRV/Fc as a vaccine [7] is maintained over the long term.

Although the ability of PRV/Fc to induce higher levels of anti-PRV antibodies is beneficial for a potential vaccine, PRV/Fc harbors the fusion protein mTR-Fc, which contains a mouse transferrin receptor-derived peptide and Fc domain of mouse IgG1. It has been reported that fusion protein of self-antigen and Fc can induce auto-reactive antibodies against the self-antigen [2, 5, 6]. We measured antibody against mouse derived A31 cell lysate by ELISA and found that a significantly higher level of anti-A31 IgG was observed in PRV/Fc immunized mice compared with normal PRV immunized mice (Fig. 3). Since no significant difference in the total amount of IgG in the serum was observed between the mouse groups immunized with PRV/Fc or with control normal PRV (data not shown), the high anti-A31 antibody titer in PRV/Fc immunized mice did not seem to be due to high levels of non-specific binding antibodies.

Properties of the antibodies induced by immunization with PRV/Fc and reacting with mouse cells: High antibody production may cause autoimmune diseases, due to autoantibodies. However, no mice immunized with PRV/Fc showed any typical clinical symptoms of autoimmune diseases (data not shown). In addition, no inflammation or disorders associated with autoantibodies were detected by histopathological analyses of the kidneys (Fig. 4), where high level TR is expressed [1]. These results indicate that the antibodies which were induced by PRV/Fc immunization and reacted with A31 cells did not have cytotoxicity.

The antibody reaction with A31 cells was further investigated by carrying out Western blot analysis using A31 cell lysate as antigen. As shown in Fig. 5, sera of mice immunized with PRV/Fc only reacted, weakly, with a protein of approximately 16 kDa. The molecular weight of the reacted protein was different from that of mTR (84 kDa). The sera of the PRV/Fc immunized mice also recognized the 16 kDa protein in RK13 cell lysate, in which PRV and PRV/Fc had been propagated. The protein in both A31 and RK13 cells was also detected by using serum from normal PRV immunized mice. In addition, an indirect immunofluorescence assay was conducted using non-fixed A31 cells, to ensure that antibody reacting mTR would be detected by Western blot analyses because the mTR antigen was denaturalized by SDS-PAGE. As shown in Fig. 6D, strong expression of mTR on A31 cells was demonstrated with an anti-mTR antibody, whereas it was not detected by serum from PRV/Fc, normal PRV immunized mice and non-treated mice (Fig. 6A, B and C).

These results indicate that the fusion protein, mTR-Fc does not induce autoantibodies against mTR.
DISCUSSION

Previously we reported that immunization with the virus particles harboring Fc induced higher levels of immune responses than normal virus particles [7]. In order to assess the practical use of the Fc harboring virus particle as a vaccine, this current study investigated any possible undesirable side-effects of immunization with the Fc harboring virus from the aspect of autoimmunity.

It is known that immunization with antigen-Fc fusion protein induces higher levels of immune responses than immunization with only the antigen [11]. The same effect has also been observed when self-derived antigens were fused with Fc, such as when the fusion protein of self Igβ and Igα antigens was fused with Fc to induce auto-reactive antibody [2, 5, 6]. These reports suggest that self-derived antigen fused with Fc could induce harmful immune responses against self antigen. In our strategy, Fc domain was expressed on the surface of RK/SK as a fusion protein with mTR [7]. The Fc was incorporated into the virus particle (PRV) after the PRV had invaded the RK/SK cells and replicated, converting it into PRV/Fc (Fig. 1). The mTR was mouse-derived antigen and it is widely expressed in mouse tissue. Therefore, the mTR-Fc molecule could also induce auto-reactive immune responses against mTR molecules, which might be harmful.

Although the immunization with PRV/Fc induced antibodies that reacted with mouse derived A31 cells (Fig. 3), the reaction with the protein of approximately 16 kDa occurred not only in A31 cells, but also in RK13 cells in which the virus had been propagated. The band patterns of these Western blot analyses, using A31 and RK13 cells, were very similar to each other. Moreover, these proteins were also detected by sera of normal PRV immunized mice. These results strongly suggest that the antibodies reacting with the mouse derived A31 cells were not specifically induced against the fusion protein mTR-Fc. Instead, it is likely that the antibodies were reacting against trace...
amounts of RK13 cell derived antigens, contained in PRV or PRV/Fc preparations, and cross-reacted with the counterpart molecules in A31 cells. In addition, no anti-mTR antibody was detected in the serum of either the PRV/Fc or PRV immunized mice, by indirect immunofluorescent assay using non-fixed A31 cells (Fig. 6). These results indicate that the immunization with PRV/Fc did not induce anti-mTR autoantibodies.

In this study, it was shown that the immunization with PRV/Fc induced higher levels of immune reactions against not only virus antigens, but also trace amounts of the protein of approximately 16 kDa derived from RK13 host cells. Although it was not confirmed whether the immunization with PRV/Fc induced antibodies reacting with authentic mouse IgG Fc and/or mTR-Fc protein, the antibodies induced by PRV/Fc immunization were not harmful to mouse tissue (Fig. 4). In addition, in this study all mice were immunized with 4 × 10⁶ pfu virus/mouse (before inactivation), a dose 100 times higher than the necessary protective dose [7]. Therefore, in terms of the practical use of the vaccine, it is believed that the normal induction levels of antibodies against RK13 will be much less than those found in this study. Thus, the cross-reactive antibodies would not be a real concern for the practical use of the PRV/Fc as a vaccine.

In this research, we have shown the safety of our newly established vaccine strategy in terms of autoimmune diseases and reconfirmed its practical value. We believe that this vaccine strategy may also be suitable for other viruses with envelope.

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REFERENCES