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LIMITED IMMUNE RESPONSE CONFERRED BY AN E2-CSFV EXPRESSING DNA VACCINE AFTER BOOSTER VACCINATION IN MURINE MODELS

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SUMMARY

To investigate if intradermal (ID) and intramuscular (IM) routes of vaccination result in a comparable expression of E2 gene of classical swine fever virus (CSFV), an established E2 DNA construct was tested in murine experimental models. Groups of six mice received three pcDNA+E2 immunisation each at weeks 0, 4 and 8 by either ID via gene gun delivery or IM. Another second batch of six mice was immunised in a similar protocol with a plasmid cocktail of pcDNA+E2 and immunomodulators (pBOOST-mIL4/mIL13). The findings showed that the IM route was better in immune induction compared to the gene gun method. Apparently, on the basis of E2 expression in skin tissues via ID, ear targeting seemed to be better than the abdominal skin approach. With respect to the kinetics of anti-E2 antibody production, the antibody was not formed after one and two months after the DNA immunisation, but reached its detection level at 10 weeks post-immunisation. Variations were observed in the ability of the E2 expression plasmids to induce immune response in murine model when the humoral and cell-mediated immunity (CMI) was determined by ELISA and DTH trials, respectively. The adopted ID and IM immunisation approaches gave distinct immune induction patterns in the presence and absence of cytokines. The findings indicate the enhancement of immune response after co-administration of these genetic immunomodulators with DNA vaccine against CSFV. However, the difference was insignificant ($P>0.05$) due to the high variance caused by the presence of non-responsive individuals.

Keywords: Classical swine fever virus, DNA vaccine, envelope glycoprotein E2, immunomodulators

INTRODUCTION

Since the E2 gene of CSFV is the major envelope glycoprotein which is exposed to the outer surface of the virion, it represents an important target for induction of immune response during infection. This protein can induce neutralising antibodies (Weiland *et al.*, 1990) and confer protective immunity in pigs (Konig *et al.*, 1995). One of the recommended vaccination approaches is using subunit vaccine. This so-called marker or DIVA (differentiating infected from vaccinated animals) vaccines in combination with sensitive and specific discriminating antibody assays would allow differentiation of infected from vaccinated animals (Van Oirschot, 1999a). When pigs are immunised with such vaccine, they only produce antibodies against E2, but not against one of the other immunogenic proteins, namely E rns. Therefore, serological tests intended to be used to differentiate infected from E2-vaccinated animals are based on the specific detection of E rns antibodies (Moormann *et al.*, 1996; Floegel-Niesmann, 2001). Therefore, any positive result implies a previous CSFV infection, whereas a negative result can either be caused by seronegative, naive pigs, or by E2-vaccinated pigs (Moormann *et al.*, 2000; Andrew *et al.*, 2000; Yu *et al.*, 2001).

In the strive for the development of an improved vaccine, a 'live' vaccine candidate with multiple markers would be beneficial to address problems regarding the immune response and vaccine status of an animal. In most cases, the direct inoculation of plasmid DNA containing ORFs with appropriate eukaryotic transcription and translation control signals, resulted in *in vivo* synthesis of a protein with identical conformation and post-translational modification to that of original protein (Hassett and Whitton, 1996). Protective immune response can be generated following inoculation of naked DNA via skin, muscle and intravenously (Johnston *et al.*, 1991). Intramuscular (IM) injection of DNA has been studied extensively for DNA vaccination (Dufour, 2001). On the other hand, gene gun (g.g) vaccination has also been shown to be effective in producing immunity (Dufour, 2001). Immunisations can be accomplished by injecting DNA in saline, or by using a gene gun to deliver DNA-coated gold beads into cells. The immune responses generated even with a single dose of DNA have the potential to elicit long lasting cytotoxic T-lymphocyte (CTL) and antibody responses (Raz *et al.*, 1994; Michel *et al.*, 1995). Therefore, the study aims to determine and assess the potential of an E2 expressing construct in inducing specific humoral and cell-mediated immunity.

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MATERIALS AND METHODS

Plasmids and monoclonal antibody

Development of E2-CSFV expression cassette has been described elsewhere (Zeenathul, 2004). E2-specific WH211 monoclonal antibodies (Veterinary Laboratories Agency-Weybridge) were used for standard immunoperoxidase assay (Zeenathul, 2004).

Preparation for mice immunisation

Female HSD mice aged 8 weeks were used. On the day prior to the experiment, the targeted region (ear, abdominal skin or hind leg) was shaved and marked. Prior to each immunisation, the mice were sedated with diethyl ether and the skin was alcohol swabbed.

For gene gun ID delivery, a hand-held gas driven microprojectile bombardment device (GENEBOY™, Budapest, Hungary) was used. The system uses high pressure carbon-dioxide gas released hand operated valve to propel the plastic macroprojectiles loaded with millions of gold or tungsten microcarriers toward target cells at high velocity. Gold was chosen instead of tungsten, because it is inert and non toxic, less traumatic, has more inertia and thus will penetrate the tissue deeper (Anderson *et al.*, 2000). The gene gun was positioned 90 degrees and 2 cm gap away from the targeted region (ear or abdominal skin). Single or dual DNA/microcarrier was shot delivered using CO_2 pressure ranging from 23 to 30 bar (350-450 psi). The depth of penetration of the gold particles is a function of several parameters, each of which can be optimised to achieve the depth required. On the first immunisation, a CO_2 pressure of 23 bar was applied and increased to 27 and 30 bar on the subsequent two immunisations, respectively. The final DNA-gold mixture was suspended in $CaCl_2$ to take advantage of its tissue transfection property.

For IM delivery, the plasmid DNA was prepared by diluting the purified DNA preparations to 2 μg/μl in PBS. Either fifty or 100 μg of plasmid/mouse was administered into the *tibialis anterior* (TA) region of the hind leg muscle using 1 ml insulin syringe (27 gauge).

Immunisation protocols

Two separate experiments were undertaken to assess the effect of route of delivery in the presence or absence of immunomodulators. In the preliminary experiment, two immunisations via i.m. route were done. A group of 6 mice was immunised with 50μg pcDNA+E2 /mouse at 4 week intervals. Heart bled was done at weeks 4 and 8 post-immunisation.

In the second experiment, both IM injection and g.g. delivery were performed in the presence or absence of immunomudulators (pBOOST-mIL4/mIL13). Both experiments were conducted simultaneously for comparison purposes. Groups of six mice received three pcDNA+E2 immunisations each at weeks 0, 4 and 8 by either IM or g.g. delivery. Another second batch of six mice were immunised in a similar protocol with a plasmid cocktail of pcDNA+E2 and pBOOST-mIL4/mIL13. One hundred and 50 μg of pcDNA+E2 and pBOOST respectively were injected in mice via the i.m. technique, while only 2 μg of pcDNA+E2 and and 1 μg of pBOOST, were introduced into mice using g.g. technique. For the g.g. method, DNA-gold was bombarded into the left ear or abdominal skin. Post-immunisation serum samples were then obtained at weeks 4, 8 and 10 by heart bleeds.

The negative control groups were either mock-inoculated or bombarded with respective concentrations of blank vectors while the positive controls were vaccinated with classical swine fever vaccine strain GPE$^-$. Three mice per group from the g.g. experiments were sacrificed after the final blood collection. The bombarded areas of the ear pinnae and abdominal skin were collected and immediately frozen in liquid nitrogen. To determine the *in vivo* expression, the tissues were cryosectioned at 10-μm thickness and mounted onto poly-L-lysine coated slides prior to immunoperoxidase staining. Other mice in each group were tested for DTH in subsequent experiments.

Delayed-type hypersensitivity reaction

T cell stimulation *in vivo* was analysed in the delayed-type hypersensitivity reaction (DTH) test by determining ear pinnae thickness. The test antigens used for DTH test were 20 μl of heat-inactivated CSFV GPE$^-$ (10 TCID50 prior to heat inactivation) and 20 μl of PBS respectively, which were injected in the right and left ear pinnae of mice. Two weeks after the final booster, test antigens were injected in the ear pinnae of sedated mice and the ear thickness was measured 48 hours post-injection with a screw gauge meter (Oditest; Germany) as previously described (Ali, 1999). Ear thickness was expressed as follows: specific ear swelling =(48 hours measurement of right ear – 0 hour measurement of right ear) - (48 hour measurement of left ear – 0 hr measurement of left ear) X 10^{-2} mm (21). Ear swelling responses at 48 hours after injection are presented as percentage of positive control (= 100%).

Antibody assays

A recombinant E2 glycoprotein pre-coated microtiter plates, from a commercially available ELISA kit (CHEKIT-CSF-SERO kit, Bommeli Diagnostics, Switzerland) was utilised to determine the presence of CSFV E2-specific antibodies in mice sera. The result for each test serum was determined as inhibition standard as follows: 10 x (median absorbance for test serum minus median

absorbance for negative pool)/ (median absorbance for positive serum minus median absorbance for the negative pool) (Siegel and Remington, 1983) and results are displayed on a scale of 0 to 10.

RESULTS

In vivo expression analysis

This study made comparison between ID and IM routes of DNA vaccination with E2 plasmid constructs. Since, ID delivery could best be performed through gene gun technique, two different skin areas (abdominal skin and ear pinnae) were targeted to make comparisons (Figure 1). Apparently, on the basis of E2 expression in skin tissues, ear targeting seemed to be better than the abdominal skin approach (Figure 2).

Immune response after vaccination with E2-CSFV DNA vaccine

All the experimental mice did not show any detectable immune response even after the 1st booster at week 4. Therefore, plasmids encoded cytokines were co-

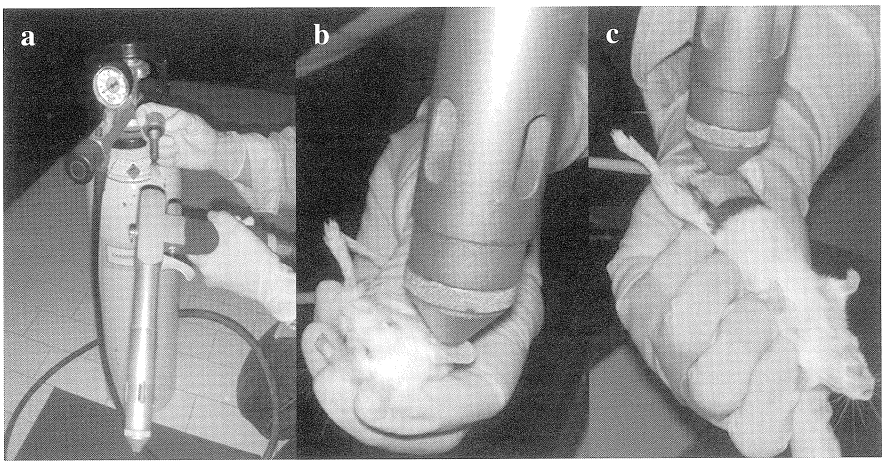

Figure 1: DNA bombardment of murine dermis using the gene gun technique. The GENEBOY™ bombardment system uses high pressure carbon dioxide gas released hand-operated valve to propel the plastic macroprojectile ('bullet') loaded with pcDNA+E2 plasmid DNA-coated-gold microcarriers toward target skin cells at high velocity.
(a) Inserting and tightening the DNA loaded bullet into the open end of the gene gun
(b) Introduction of DNA into the ear pinnae; and (c) ventral abdominal skin

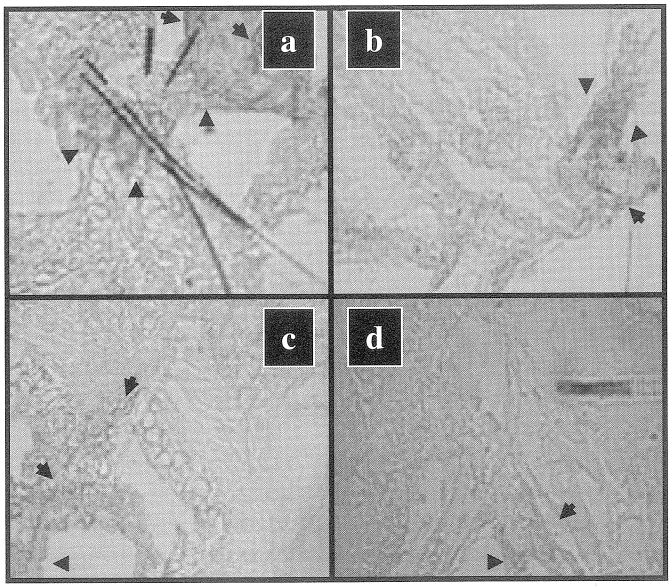

Figure 2: Expression of the DNA construct of the E2 expression cassette in the skin of the mouse. Tissues of the cryosected ear pinnae (a, b, c, d) indicated expression of the E2 protein (brown discoloration) upon immunoperoxidase staining (arrows). Original magnification at 1000X.

delivered in the subsequent study. Administration of IL4/IL13 encoded plasmids was done in increased concentration of E2 encoded plasmids in the second experiment. Surprisingly, immune response remained undetected even after increasing the plasmid concentration in the second experiment. An immune induction was obtained following the 3rd IM immunisation in the presence of immunomodulators (Figure 3). Similar to IM immunisation, mice only developed an antibody response after the 3rd immunisation. All the experimental groups showed variable immune induction regardless of the presence and absence of immunomodulators. There was no significant difference between the groups because of the low number of animals and high variability among the animals. The findings showed that IM route was better in immune induction compared to the g.g. method. With respect to the kinetics of anti-E2 antibody production, the antibody was not formed after one and two months after the DNA immunisation, but reached its detection level at 10 weeks post-immunisation (Figure 3).

T cell stimulation

Next, it was assessed whether immunisation with E2 plasmid in the presence or absence of cytokines elicited DTH type cell mediated response. The findings indicate that the E2 envelope gene, encoded DNA plasmids have the potential of giving rise to a potent cell-mediated

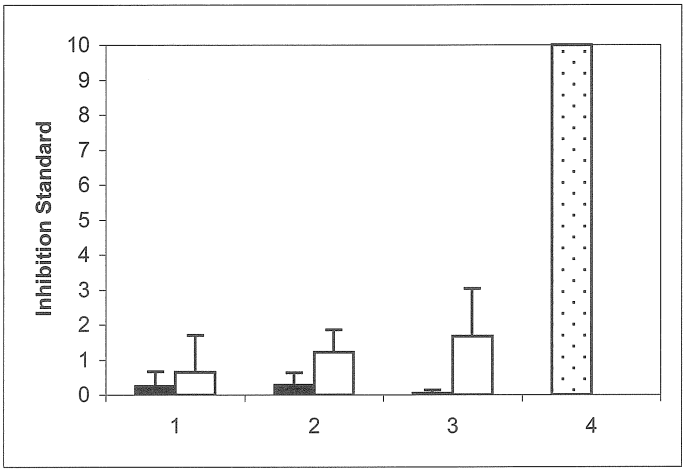

Figure 3: Detection of E2 specific antibody in mice. Groups of mice were immunised with E2 expression plasmids via intramuscular (IM) (group 3) or gene gun (g.g.) approach through bombardment onto abdominal skin (group 1) or ear pinnae (group 2) in the presence (white) or absence (black) of cytokines (IL4/IL13). The positive control group (dotted) was immunised through IM route with GPEÉ vaccine virus.

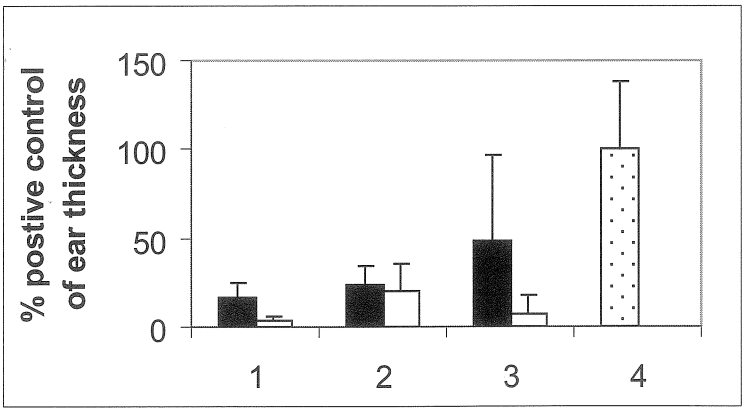

Figure 4: DTH response induced by the heat-inactivated CSFV. Groups of mice were immunised with plasmid encoding E2 gene of CSFV in the presence (white) or absence of (black) cytokine encoded plasmid, administered via IM (group 3) or gene gun, through bombardment onto abdominal skin (group 1) or ear pinnae (group 2). The positive control group (dotted) was immunised with GPE⁻ vaccine virus. Two weeks following the 2nd booster, these mice were injected with 20 µl of heat-inactivated CSFV GPE⁻ (10 TCID50) or PBS into the right or left ear pinnae, respectively. The ear swelling was measured 48 hr post-inoculation using a micrometer and the difference in ear thickness (× 10^2 mm) between the two ears is presented as percentage of positive control.

immune response by inducing a readily detectable delayed-type hypersensitivity (DTH) response to an inactivated CSFV. Two weeks after the 2nd booster immunisation, when heat-inactivated CSFV GPE$^-$ virus was injected intrapinnally, it evoked a variable DTH response in mice immunised with E2 plasmid (Figure 4) at 48 hour post-inoculation. The control ear did not show any DTH response. Findings evidenced that DTH responses were greater in the absence of the of T helper-2 (Th2) type cytokines, likewise when antibody production is increased, the DTH reaction is suppressed. However, the present findings were not sufficient to support the fact ($P>0.05$).

DISCUSSION

DNA vaccine strategies can differ greatly, with significant effects on the outcome of immunisation (Doria-Rose and Haigwood, 2003). Inoculation of mice with pcDNA+E2 elicited an immune response. On the basis of E2 expression via ID route, ear targeting seemed to be better than abdominal skin approach as the microprojectiles can more readily penetrate the thin epidermis of the ear and introduce DNA into more cells within the dermis, revealing distinct E2 gene expression.

The present immunisation regimens addressed considerations of doses, timing of doses, genetic adjuvants, and routes of vaccination. In addition, humoral immune response was reported to be enhanced when animals received DNA primed-DNA booster (Rollier *et al.*, 2000). Booster immunisations seemed promising in this study. Other studies had also indicated that manipulation of these parameters could alter immune responses that differed in magnitude, quality and balance of cellular and humoral responses, thus providing a further enhancement for DNA immunisations (Doria-Rose and Haigwood, 2003). Despite those manipulations, the results showed an unexplained slow responsiveness towards immune induction. For example, pigs immunised by DNA vaccine against pseudorabies (PrV) did not reveal humoral immune response at day 5 post vaccination but antibodies were produced at low levels 7 days after PrV challenge (Dory *et al.*, 2007). Perhaps there are other unknown critical reasons, probably, at the level of plasmid itself. One neglected aspect in efforts to achieve the highest level of transgene expression, is plasmid mutation or instability in host cells (Ratel and Wion, 2003). The problem of mutation frequency in plasmid DNA transfected into mammalian cells had been previously reported (Ratel and Wion, 2003) and might be of paramount importance when the gene corresponds to an aberrant protein function following the mutation. Liposome based transfection ("Lipofectin") is typically more efficient in introducing DNA into cells than calcium-based tranfection methods (Felgner *et al.*, 1987) and this might be another possible reason for the low levels of protein expression resulting from a low rate of DNA transfection in the dermis.

The direct *in vivo* inoculation of E2 encoded plasmid DNA to raise immune responses is preferred because it allowed the host cells to express the immunising proteins without the need for protein purification. Variations in the ability of the expression plasmids to induce immune response in murine model were observed when the humoral and cell-mediated immunity (CMI) were determined by ELISA and DTH trials, respectively. The adopted g.g. and IM immunisation approaches gave distinct immune induction patterns in the presence and absence of cytokines. Immune responses can be modulated by the cytokines, which is an important manifestation of T cell-mediated immunity (Cher and Mossman, 1987; Flo *et al.*, 2000). In fact, cytokines control the immune response by influencing and changing the balance of T helper-1 (Th1) and Th2 cells (Roman *et al.*, 1997). The findings indicated the enhancement of immune response after co-administration of these genetic immunomodulators with DNA vaccine against CSFV. However, the difference was insignificant ($P> 0.05$) due to the high variance caused by the presence of non-responsive individuals. The findings of CMI response were similar to the previously reported DTH response against HIV envelope protein encoded expression plasmids (Okuda *et al.*, 1995).

Our observations indicate that the IM technique is a superior route of immunisation to induce immune response compared to the g.g. approach. The effect of the DNA delivery route on immune induction has been constantly analysed. In pigs, IM injections of a DNA vaccine against CSFV provided higher levels of antibodies than the g.g. route (Andrew *et al.*, 2000), whereas ID injections of PRV plasmid induced a higher immune response than IM injections (Van Rooij *et al.*, 1998). Conversely, in rabbits, g.g. delivery of the E1 and E2 genes of cotton-tail rabbit papillomavirus (CRPV) provides complete protection, whereas IM injections were not effective (Han *et al.*, 2000). In dogs, IM injection of a DNA vaccination against rabies virus (RV) resulted in a higher and more durable antibody response than that obtained by ID injection (Osorio *et al.*, 1999), whereas in cats, ID injections of the same plasmid elicited a higher frequency of seroconversion than IM administration. Both techniques could produce both cellular and humoral response at variable levels (Raz *et al.*,1996). Therefore, these results were not dependant on the presence of antigen presenting cells (APVs) since the dermis is rich with those cells (Tuting *et al.*, 1998). Thus, the efficacy of DNA vaccines varies not only according to the route of delivery, but also to animal species and pathogen.

Nevertheless, the findings could also be attributed to the technical approach, in the sense that more parameters had yet to be optimised. In the case of the gene gun experiment, the reduced level of successful DNA

bombardment could probably be the reason for the reduced rate of transfection and subsequent expression. Post g.g application, some of the DNA-gold-CaCl mixture seemed to be retained at the outer skin, rendered impenetratable upon bombardment. The depth of penetration depends on the pressure by which the gold particles are accelerated, as well as their size (Anderson *et al.*, 2000). Larger particles are heavier, and gain more kinetic energy, therefore, they penetrate deeper but can cause more tissue damage (Anderson *et al.*, 2000). The present study utilised small gold particles (0.6 μ) which explained the observation. Therefore, a slightly larger gold size such as 1μ might perhaps help solve the problem of incomplete DNA penetration.

The study evidenced immune inductions and E2 expressions, which could be used to corroborate the presentation of normally processed proteins to the immune systems. It suggests that the membrane expression of the E2 protein is important for effective antigen presentation. This process is essential to raising immune responses against the native forms of the protein (Hansson *et al.*, 2000). Although the immune response to the inserted E2 gene product received only a preliminary examination, the techniques for delivery of the current DNA construct warrant good optimisation. Eventually, this opens the possibility of exploring the potential of the DNA vaccine to be developed as marker vaccines against CSF or to be incorporated into various viral vectors.

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