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Humoral and cellular immune response in mice induced by the classical swine fever virus E2 protein fused to the porcine CD154 antigen

Yusmel Sordo[a,*], Marisela Suárez[a], Rosalina Caraballo[b], Talía Sardina[a], Emma Brown[b], Carlos Duarte[c], Joanna Lugo[a], Lázaro Gil[d], Danny Perez[a], Ayme Oliva[a], Milagros Vargas[a], Elaine Santana[a], Rodolfo Valdés[e], María Pilar Rodríguez[a]

[a] Animal Biotechnology Department, Center for Genetic Engineering and Biotechnology, POBox 6162, Havana 10600, Cuba
[b] Animal Breeding Department, Center for Genetic Engineering and Biotechnology, POBox 6162, Havana 10600, Cuba
[c] Pharmaceutical Department, Center for Genetic Engineering and Biotechnology, POBox 6162, Havana 10600, Cuba
[d] Vaccine Department, Center for Genetic Engineering and Biotechnology, POBox 6162, Havana 10600, Cuba
[e] Monoclonal Antibody Production Department, Center for Genetic Engineering and Biotechnology, POBox 6162, Havana 10600, Cuba

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ABSTRACT

The development of subunit vaccines against classical swine fever is a desirable goal, because it allows discrimination between vaccinated and infected animals. In this study, humoral and cellular immune response elicited in inbred BALB/c mice by immunization with a recombinant classical swine fever virus (CSFV) E2 protein fused to porcine CD154 antigen (E2CD154) was assessed. This model was used as a predictor of immune response in swine. Mice were immunized with E2CD154 emulsified in Montanide ISA50V2 or dissolved in saline on days 1 and 21. Another group received E2His antigen, without CD154, in the same adjuvant. Montanide ISA50V2 or saline served as negative controls for each experimental group. Animals immunized with 12.5 and 2.5 μg/dose of E2CD154 developed the highest titers (> 1:2000) of CSFV neutralizing antibodies. Moreover, CSFV specific splenocyte gamma-interferon production, measured after seven and twenty-eight days of immunization, was significantly higher in mice immunized with 12.5 μg of E2CD154. As a conclusion, E2CD154 emulsified in Montanide ISA50 V2 was able to induce a potent humoral and an early cellular immune response in inbred BALB/c mice. Therefore, this immunogen might be an appropriate candidate to elicit immune response in swine, control CSF disease and to eliminate CSFV in swine.

1. Introduction

Classical swine fever (CSF) is a highly contagious viral disease, which causes major losses in swine production. The most frequent clinical signs and lesions of the disease are fever, hemorrhage, ataxia and immunosuppression [1]. The CSF has a worldwide distribution with high prevalence in East and Southeast Asia, Eastern Europe, South America, Central America and some Caribbean countries [2,3]. Although Western European countries are considered CSF free; some periodical outbreaks are produced causing great economic losses as well [3–6].

The causative agent of the disease is CSF virus (CSFV), an enveloped, single-stranded RNA virus from the *Pestivirus* genus and *Flaviviridae* family [1,7,8]. The mode of transmission is oral-nasal through direct or indirect contact with infected animals or the consumption of virus contaminated food [9].

CSF traditional control has been the immunization of swine with CSFV attenuated vaccines. This type of vaccines develops a protective immune response capable of withstanding high dose of confrontation with pathogenic strains of CSFV [10–12]. Nonetheless, attenuated vaccines induce an immune response pattern similar to those observed in those animals infected with CSFV, implying that there is not chance to distinguish by serological methods between vaccinated and CSFV infected animals [13]. As a consequence, vaccination of animals with attenuated virus strains might have a negative impact on exportation of swine and swine derived products [14]. On the other hand, virus strains used in the vaccination programs could impose a selective pressure on wild virus strains, inducing mutations in the wild virus strains [8,15].

For these reasons, the use of attenuated vaccines has been forbidden in Europe, where CSF outbreaks are usually controlled by movement control and stamping out of infected or suspected swine [14]. This policy leads to the killing of a large number of uninfected swine, which

* Corresponding author.
E-mail address: yusmel.sordo@cigb.edu.cu (Y. Sordo).

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not only has a negative economic impact, but is also questionable from an ethical point of view [16].

Therefore, and looking for a similar immune response to those observed in animals vaccinated with attenuated CSFV vaccines, new protein subunit vaccines have been developed. However, so far, only two subunit protein vaccines against CSFV have reached the commercial stage: BAYOVAC® (Bayer, Germany) and Porcilis® Pesti (Intervet, The Netherlands). These two vaccines are based on the E2 glycoprotein of the virus envelope expressed in baculovirus. Although both vaccines fulfilled the DIVA concept [17], they were found to be less effective than live attenuated vaccine; do not induce an early response, and confer only incomplete protection in vertical transmission studies. That is why their use is only recommended in the eradication phase of the disease [18–21].

Our group developed a protein subunit vaccine candidate, containing the E2 protein of CSFV expressed in HEK-293 cells and fused to the porcine CD154 antigen. This E2 protein exhibits the same amino acid sequence and displays similar posttranslational modifications to the E2 viral counterpart. On the other hand, the CD154 (CD40L) is an immune system stimulating protein also called “molecular adjuvant”. It is member of the tumor necrosis factor ligand family, and has a central role in the development and regulation of adaptive immune response in mammalian and avian species [22]. It is an integral membrane glycoprotein expressed on the surface of activated T cells [23], B cells [24], basophils and mast cells. This molecule has been defined as the most important costimulatory factor for the activation of antigen presenting cells [25–27]. Its receptor (CD40) belongs to the same TNF superfamily, and it is a surface protein of B cells, dendritic cells (DCs), macrophages, Langerhans cells, epithelial cells, endothelial cells and fibroblasts [25]. The binding of CD154 to CD40 on the surface of B cells stimulates the cell proliferation, adhesion and differentiation. CD40 participation leads to the clonal expansion of B cells, germinal center formation, isotype change, affinity maturation and the generation of long-lived plasma cells [23]. CD40 antibodies that mimic the endogenous CD40-CD154 interaction have been shown to exhibit potent adjuvant effects when bound to different antigens [28]. Based on these properties CD154 has been successfully used as a molecular adjuvant by several authors [25,28,29].

Following the same line of thought here we hypothesized that the combination of E2 with CD154, used as a molecular adjuvant, might enhanced either the humoral or the cellular immune responses in animals. Since experiments in swine are expensive and cumbersome; the assessment of this hypothesis was done in inbred mice as preliminary evidence before proceeding to demonstrate the immunogenic potential of E2CD154 vaccine candidate in swine.

2. Materials and methods

2.1. Immunogen

The active ingredient of the subunit vaccine candidate is a chimeric protein formed by the fusion of the extracellular region of E2 glycoprotein of CSFV and the extracellular segment of swine CD154 molecule. The E2CD154 was expressed and isolated from HEK-293 cell line with more than 90% of SDS-PAGE purity and formulated at 12.5 and 2.5 μg/dose in saline or in Montanide ISA50 V2 (SEPPIC, France) in an ULTRA-TURRAX T25 basic homogenizer (IKA Works INC., NC), at a proportion of 40% oil phase and 60% aqueous phase. The E2his antigen was produced and purified from the milk of adenoviral transduced goats as previously described. E2his glycoprotein, diluted in phosphate buffer, was formulated as a water-in oil emulsion with Montanide ISA50 V2 (SEPPIC, France) as previously described [30].

2.2. Experimental animals and immunization protocol

Eight week-old female BALB/c mice of 18–20 g of weight purchased by the National Center for Animal Breeding (CENPALAB, Cuba) were used in the study. All animals were intraperitoneally immunized with disposable syringes and 21 × 1″ needles following a vaccination schedule of two administrations every 21 days. The study code was 5SIE1407. It was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) according to the regulation No. 64–2013 from CECMED (Centro para el Control de Medicamentos de Cuba) entitled: Lineamientos para la Constitución y Funcionamiento de los Comités Institucionales para el Cuidado y Uso de los Animales de Laboratorio (CICUAL) [31].

2.3. Experimental design

2.3.1. Immunogenicity of E2CD154 with or without adjuvant

Seven experimental groups were included. All animals received the immunogen in a volume of 200 μL. Mice from groups 1 and 2 (n = 15) received 12.5 and 2.5 μg of E2CD154 emulsified in Montanide ISA50 V2, respectively. Animals from groups 3 and 4 (n = 15) were given doses of 12.5 and 2.5 μg of E2CD154 in saline, respectively; animals from group 5 (n = 15) were immunized with 12.5 μg of E2his emulsified in Montanide ISA50 V2. Finally, mice from groups 6 and 7 (n = 15) received a Montanide ISA50 V2 emulsion or saline respectively (placebo controls). Serum samples were taken and processed at days 1, 14, 21 and 35 after immunization to detect neutralizing antibodies against the CSFV. To analyze the cellular immune response induced by E2CD154, 5 animals from groups 1 and 5 were euthanized with pentobarbital (200 mg/kg) at days 7 and 28 after the first immunization. All animals were immunized by intraperitoneal route. The total number of animals used was 105.

2.3.2. Potency test of E2CD154 emulsified in Montanide ISA50 V2

Six experimental groups were included (8 mice per group). All immunogens were applied by intraperitoneal route in a volume of 200 μL. Mice in groups 1 to 6 received 2.5, 1.25, 0.625, 0.3125, 0.1562 and 0 μg of E2CD154, respectively. Blood samples were taken by ophthalmic venous sinus punction and processed at days 1 and 35 after immunization to detect neutralizing antibodies against the CSFV. The total number of animals used was 48.

2.4. NPLA for detection of neutralizing antibodies against CSFV

The neutralizing peroxidase-linked assay (NPLA) was conducted as described in the OIE manual [32] for the detection of CSFV neutralizing antibodies. Serum samples were screened for the ability to neutralize 100 $TCID_{50}$ of CSFV produced in PK15 cells. The Margarita strain isolated in Cuba in 1958 was used. The virus was kindly provided by the National Center for Animal and Plant Health, Mayabeque Cuba.

2.5. Mouse splenocytes isolation and in vitro stimulation with CSFV antigens

Mice were euthanized with pentobarbital (200 mg/kg). Each group was composed by 15 animals. Five mice from groups 1 and 5 were euthanized at days 1 and 28 post vaccination. Spleens were aseptically removed and placed in 5 mL of 150 mM PBS, 5% fetal bovine serum, penicillin (100 IU), streptomycin (100 μg). Next, spleens were perfused with 5 mL of the same solution using a 5 mL syringe and 21G needle. Cell suspensions were then centrifuged at 1500 rpm for 10 min. Subsequently, cell pellets were gently agitated and 3 mL of red blood cell lysis buffer were added. The mixture was incubated for other 5 min at room temperature. Later, two consecutive washes with 10 mL of PBS, 5% fetal bovine serum, penicillin (100 IU), streptomycin (100 μg) were done and the respective cell suspensions were centrifuged again at 1500 rpm for 10 min. Cells were counted to be seeded in 96 well-plates at 200 × 10^3 cells/well in RPMI 1640, 5% fetal bovine serum, 100 IU penicillin and 100 μg streptomycin. Two wells with cells