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Chemokine CCL20 plasmid improves protective efficacy of the Montanide ISA™ 206 adjuvanted foot-and-mouth disease vaccine in mice model

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ABSTRACT

This study aimed to investigate the chemokine CCL20, a macrophage inflammatory protein-3 alpha, for adjuvant potential in inactivated foot-and-mouth disease (FMD) vaccine. Groups of mice were injected intramuscularly with either murine CCL20 DNA or CCL20 protein two days ahead of the immunization with Montanide ISA206 adjuvanted inactivated FMD vaccine and humoral and cellular immune responses were measured in post-vaccinal sera. We demonstrated that the mice immunized with CCL20 plasmid plus FMD vaccine showed earlier and significantly ($p < 0.05$) higher neutralizing antibody responses compared to the mice vaccinated with CCL20 protein plus FMD vaccine. In fact, CCL20 as a protein did not show any adjuvant effect and the immune responses induced in this group were comparable to that of the mice vaccinated with FMD vaccine alone. All the vaccination groups showed serum IgG1 and IgG2 antibody responses; however, the mice vaccinated with CCL20 plasmid plus FMD vaccine showed significantly ($p < 0.05$) higher IgG1 and IgG2 responses and the responses remained high at all-time points post vaccination, although not always statistically significant. Upon restimulation of the vaccinated splenocytes with the inactivated FMD viral antigen, significantly ($p < 0.05$) higher IFN-γ and IL-2 levels in culture supernatants were found in animals vaccinated with the CCL20 plasmid plus FMD vaccine, which is indicative of the T_H1 type of cellular immunity. On challenge with the homologous FMD virus on 28th day post immunization, CCL20 plasmid plus FMD vaccine showed complete protection (100%) while animals immunized with CCL20 protein plus FMD vaccine or FMD vaccine alone showed 66% protection. In summary, we show that prior injection of CCL20 plasmid improved protective efficacy of the inactivated FMD vaccine and thus offers a valuable strategy to modulate the efficacy and polarization of specific immunity against inactivated vaccines.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious and economically important disease of domestic and wild cloven-hoofed animals. The disease mainly affects cattle, sheep, goats and pigs, and is characterized by fever, lameness, and vesicular lesions on the tongue, feet, snout, and teats [1]. FMD affects directly or indirectly the livelihood of millions of small scale farmers and is considered one of the biggest impediments to international trade. The disease causing agent, FMD virus (FMDV), is a positive sense RNA virus belonging to the genus *Aphthovirus* in the family *Picornaviridae* [1]. FMDV is characterized by high antigenic variations that are reflected into seven distinct serotypes and numerous subtypes. Infection with one serotype does not confer cross protective immunity against other serotypes, and that often complicates the disease control programs [2,3]. The disease has been successfully controlled by vaccination through use of binary ethylenimine (BEI) inactivated cell culture grown virions formulated in Al(OH)3/saponins or Montanide-based oil adjuvants [4]. These vaccines are effective in reducing clinical disease and have been successfully used in several FMD control programs. However, these vaccines induce low cell-mediated immune (CMI) responses and shorter duration of immunity (<6 months) (reviewed in [4]), To elicit superior immune responses and to increase the immunogenicity

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of currently available FMDV vaccines, in this study, we explored murine chemokine ligand 20 (CCL20) as an adjuvant in Montanide ISA206 adjuvanted inactivated FMD vaccine in murine model.

Chemokines are a group of very small chemotactic cytokines that play important role in the development of innate and adaptive immunity by regulating inflammatory reactions, lymphocyte trafficking to the site of infection or vaccination, and immune cell differentiation [6,7]. Chemokines not only guide immune effector cells to sites of infection or vaccination, but they also facilitate interactions between innate and adaptive immune cells, thus shaping and providing the necessary environment for the development of effective adaptive immunity [7]. CCL20 chemokine, which is also known as liver activation regulated chemokine (LARC) or macrophage inflammatory protein (MIP)-3-alpha, is known to interact with the CCR6 receptor present on the immature dendritic cells (DCs) that selectively recruits these cells to the site of injection or antigen entry [8]. The DCs are unique and potent professional antigen presenting cells (APCs) with ability to prime effective antigen-specific adaptive immune responses and permit the establishment of immunological memory [9]. DCs encounter the foreign antigens in the peripheral tissues, capture and process them, and then migrate to secondary lymphoid organs, wherein processed foreign antigens are presented to the naïve T cells necessary for the initiation of adaptive immune responses [10]. Besides DCs, CCR6, the functional receptor for CCL20, is also expressed in lymphocytes, neutrophils and lymphoid tissues, including the lymph node and the spleen [8]. A study by Schutyser et al. shows that CCL20 and CCR6 interaction drives the chemoattraction of DCs, effector/memory T cells and B cells at mucosal surfaces under physiological and inflammatory conditions [8]. Considering the potential CCL20 to recruit innate and adaptive immune cells to the site of immunization or infection, the aim of this work was to explore whether injection of either CCL20 plasmid or CCL20 protein ahead of the immunization with ISA™206 adjuvanted inactivated FMD vaccine could improve FMDV-specific protective immune response in murine model. We show that CCL20 plasmid has improved FMDV-specific both humoral and CMI responses compared to the CCL20 protein, which failed to show any adjuvant effect. We conclude that CCL20 as a DNA has potential to elicitate potent antigen-specific immunity and can be, thus, incorporated in vaccine formulations to augment immune responses against weak immunogenic inactivated vaccines.

2. Material and methods

2.1. Virus and cell culture

FMDV vaccine strain 'O/IND R2/75' maintained at the Immunology Lab, FMD QCQA Unit, Bangalore, India was propagated in Baby Hamster Kidney (BHK)-21 clone 13 (Glasgow) at 37 °C. The BHK-21 cell line was cultured in Dulbecco's modified minimum essential medium (DMEM; Sigma, USA), supplemented with 10% fetal calf serum (FCS, Hyclone, USA) and 25 mM HEPES (Sigma Aldrich, USA).

2.2. Preparation of inactivated FMD vaccine

Vaccine virus strain of FMDV O/IND/R2/75 was used for antigen production in BHK-21 cell monolayer using standard procedure as described elsewhere [11]. The antigen was diluted in PBS (pH 7.4) to a required concentration and then added to the oil-adjuvant, Montanide ISA-206 in a 50:50 (w/w) ratio. The mixture was stirred at a low shear rate (300 rpm) for 10 min at 30 °C in an auto-mix blender (REMI laboratory instruments, India) to form double emulsion (water-in-oil-in-water). The inactivated FMD vaccine formulated with Montanide ISA-206 contained 1 μg of 146S virus particles of antigenic mass per dose.

2.3. Construction of pcDNA-CCL20 plasmid construct and confirmation of protein expression in mammalian cells

The gene encoding murine CCL20 chemokine was synthesized (Basil BioSolutions, Bangalore) based on the sequence available in the NCBI database (Accession No. NM016960.2). The gene was amplified using gene specific primers by a polymerase chain reaction (PCR) based on the protocol described elsewhere [12]. The amplified PCR product was cloned into the pcDNA3.1 mammalian expression vector (Thermo Fisher Scientific, USA) at *BamHI/XhoI* sites and the recombinant clones were confirmed by colony PCR and restriction enzyme (RE) analysis. For visualization of CCL20 DNA expression in BHK-21 cells, green fluorescent protein (GFP) gene was cloned downstream to the CCL20 gene in pcDNA3.1 vector. The resultant plasmid pcDNA-CCL20-GFP was subsequently transfected into BHK-21 cells using Lipofectamine (Catalog# 11668019, Thermo Fisher Scientific, USA) as per the manufacturer's instructions. The mouse recombinant CCL20 protein used in this study was commercially purchased (catalog no. #760-M3).

2.4. Mice immunization and challenge studies

Healthy male C57Bl/6 mice aged between 6 and 8 weeks were purchased from Indian Institute of Science (IISc, Bangalore, India). The mice were maintained under standard conditions, and provided with pathogen and antibiotic free food and water *ad libitum*. All animal experiments were carried out at Animal Isolation Unit, Immunology Lab, FMD QCQA Unit FMD, IVRI, Bangalore, in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

A total of 60 mice were used in this study. Animals were randomly divided into four groups (n = 15) and the animals in groups 1 and 2 were first primed with 50 μg of pcDNA-CCL20 plasmid and 50 μg of CCL20 protein, respectively. After 2 days post-priming, groups 1 and 2 received Montanide ISA-206 adjuvanted inactivated FMD vaccine at the same injection site, while groups 3 and 4 received Montanide ISA-206 adjuvanted inactivated FMD vaccine alone and 50 μl of sterile PBS, respectively. Animals in the vaccine groups were immunized with 50 μl containing 1 μg of their respective vaccine intramuscularly into the tibialis muscle using 26 gauge needles. Blood sampling was done thereafter weekly to assess the levels of neutralizing antibodies and isotype-specific IgG1 and IgG2 responses in serum.

Four weeks post-immunization, all the mice were challenged intraperitoneally with 10^4 $TCID_{50}$ of homologous FMDV. The mice were analysed for the presence of viremia and the protection was assessed as per the method described in Quattrocchi et al. [13]. Briefly, blood samples collected after 48 h post-challenge were serially diluted in DMEM and the dilutions were spread on BHK-21 cell monolayers for 30 min in 96 well plate. Thereafter the plate was washed with sterile PBS and fresh DMEM with 2% fetal calf serum was added and the cells were incubated at 37 °C for 48 in a 5% CO2 incubator. An animal was considered positive if the cell layer did not present CPE after a blind passage. The viral titers were calculated by Reed and Muench method [14]. The percent protection was calculated as P% = (number of protected mice/number of challenged mice) × 100.

2.5. Serum neutralization test

Titres of neutralizing antibodies against FMDV O/IND/R2/75 virus were measured in serum samples by micro-neutralization assay as described previously [11].

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