Use of inactivated *E. coli* enterotoxins to enhance respiratory mucosal adjuvanticity during vaccination in swine

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Abstract

In order to augment responses to respiratory vaccines in swine, various adjuvants were intranasally co-administered with a Foot-and-Mouth Disease Virus (FMDV) antigen to pigs. Detoxified *E. coli* enterotoxins LTK63 and LTR72 enhanced antigen-specific mucosal and systemic immunity, demonstrating their efficacy as adjuvants for non-replicating antigens upon intranasal immunization in swine.

Body

Most pathogens initiate infections through contact with the mucosal surfaces of their hosts, thereby leading to colonization of the epithelium and/or invasion of tissues. Vaccination strategies that induce the production of mucosal immunity are desirable as this approach can reduce the contact of pathogens with epithelial cells and possibly prevent dissemination to peripheral sites of the body. This is particularly important in the respiratory tract of animals as many of the potent innate barrier defenses present in the digestive system (e.g. low stomach pH, digestive enzymes etc.) are not found in the airways. Given that the mucosae are inherently tolerogenic (1), the use of adjuvants is essential to induce robust responses for non-replicating mucosal vaccines (4). Unfortunately, the efficacy of many adjuvants in domestic animals (including pigs) has not been thoroughly tested, particularly for vaccines administered via the intranasal route (IN).

The best characterized mucosal adjuvants are the heat-labile enterotoxin (LT) produced by *Escherichia coli*, and the closely related cholera toxin (CT) elaborated by *Vibrio cholerae*. Regrettably, these toxins are unsafe for use in humans (6), which dramatically limits their use in human and veterinary vaccines. To address this issue, detoxified mutants of LT, known as LTR72 and LTK63, were previously generated and have minimal residual toxicity (5, 8), but retain mucosal adjuvant activity in some species of animals (10). Recently, several other adjuvant candidates have been developed in an attempt to elicit more potent mucosal immune responses. The interaction between bacterial CpG DNA and TLR 9 has made this motif a promising candidate for boosting both systemic and mucosal immunity. Another adjuvant that has been shown to augment mucosal and systemic immune responses is chitosan, a polysaccharide derived from the exoskeleton of crustaceans. More recently, chitosan nanoparticles harboring CpG motifs induced mucosal and systemic immune responses in mice (3, 11).

In this work, four mucosal adjuvants (wild-type CT (20μg), LTK63 (100μg), LTR72 (100μg), and CpG/chitosan (200μg); manufacturer’s recommended dosages) were tested to assess their efficacy upon co-administration with a model FMDV antigenic peptide via the intranasal route. Groups of five female Yorkshire pigs (6-weeks old; John Correy, Scotland, CT) received the mucosal adjuvants admixed with a peptide derived from FMDV serotype O1-BFS VP1 G-H loop (which contains at least one important neutralizing epitope of the virus (9)). This epitope is referred to as the “TCA” peptide, representing a well-conserved VP4 T helper cell epitope, VP1 site C, and VP1 site A epitopes. Animals were intranasally inoculated with 100μg of TCA peptide (reconstituted in 400μl of water, the volume given to each animal, Genemed Synthesis Inc., South San Francisco, CA) at weeks 1, 2, 3 and 5, with a parenteral boost given at week 4 with MPL+TDM+CWS RIBI Adjuvant System (100 μg, administered intramuscularly (IM), Sigma-
Aldrich, St. Louis, MO). The parenteral boost was included to augment serum antibody and virus neutralization titers since mucosal immunity alone may not adequately control viral infection/dissemination (7). Additionally, control groups included pigs sham immunized IN with chicken ovalbumin (OVA) plus RIBI adjuvant (Sigma Aldrich, St. Louis, MO) at week 0, with a week four IM boost of OVA and RIBI, or TCA peptide plus RIBI adjuvant IM at weeks 0 and 4. Even though CT retains toxicity, it has been shown to be efficacious when administered IN to pigs and we thus utilized this adjuvant as a “gold standard” for comparative purposes.

Serum samples were collected for assessment of anti-TCA peptide IgG responses, as measured by ELISA (previously described, (2)), and the concentration was calculated using the method described by Barrette et al. (2) (statistical significance for all analyses were determined using a one-way ANOVA with post-hoc multiple pair-wise comparisons by Fisher’s LSD test). A negligible IgG response was observed in all IN immunized groups prior to week 4; however, the IM-TCA group did produce a notable response at week 3 (data not shown). Animals IN inoculated with CT, LTR72, LTK63 or IM-TCA produced statistically significant IgG anti-peptide responses by week 4 relative to those inoculated with OVA or CpG/Chitosan (figure 1A) and this tendency was observed throughout the remainder of the experiment (LTR72 inoculated animals were not significant at week 5 but the trend could still be observed (p=0.1 when compared with OVA or CpG/Chitosan) and responses were again significant at week 6). Interestingly, responses were not different amongst the groups producing a significant IgG response, with the exception of IM-TCA and LTR72. Thus, LTR72 and LTK63 appears to induce as vigorous a serum anti-TCA peptide IgG responses as the CT adjuvanted group. It is important to note that the week 4 parenteral booster immunization of mucosally primed animals did not seem to augment immunity, indicating that IN immunization alone was primarily responsible for the observed antibody responses. In order to assess the functional capabilities of the humoral antibody responses, FMDV plaque reduction assays were conducted in order to determine virus neutralization titers (VNT). No VNT were observed at week 4 in any group; however, by the end of the study significantly higher VNT were observed in animals inoculated with LTK63 than OVA or CpG/Chitosan (figure 1C). Also, animals inoculated with LTR72 trended towards significance when compared to OVA (p=0.07) or CpG/Chitosan (p=0.05), and a trend was also observed in animals inoculated IM with TCA when compared with OVA (p=0.07) or CpG/Chitosan (p=0.05). These data indicate that mucosal immunization was as effective as IM inoculation for generating serum IgG antibodies, but IM boosting may be necessary to induce serum virus neutralization activity.

Anti-TCA peptide IgA responses (Figure 1B) were measured by ELISA from nasal wash samples collected from pigs and are reported as the geometric mean titer. Secreted IgA antibodies were evident by week 4 in pigs receiving the CT and LTR72 adjuvants when compared to all other groups, and animals inoculated with LTK63 had significant responses when compared to those receiving IM-TCA or OVA at weeks 5 and 6. Animals vaccinated with CpG/Chitosan exhibited a trend towards significance at week 5 when compared to OVA or IM-TCA (p=0.07 and p=0.06, respectively) and attained statistical significance by week 6, although responses were notably lower than those observed with the other mucosal adjuvants. No significant IgA responses were detected in animals inoculated with OVA or IM-TCA at any time points. Antigen-specific IgA antibody secreting cells (exceeding 50 cells/million by ELISpot) were
observed in the nasal mucosal tissues of some CT and LTr72 inoculated pigs, indicating that at least some of the mucosal immune responses resulted from plasma cells located in the nasal mucosa (data not shown).

Taken together, the LT mutants used as adjuvants in this study were effective for inducing systemic and mucosal immunity to an antigenic FMDV peptide in the respiratory tract of swine, whereas CpG/chitosan was less efficient. Even though the LT mutants showed efficacy in this experiment, modification of the approach is necessary to improve the time-to-immunity and to increase the magnitude of the mucosal response. Based on the delay of roughly four weeks, it appears that adjustments in dose, adjuvanticity, delivery, antigen, or frequency of administration may be needed to optimize mucosal immune responses in the respiratory tract of pigs to pathogens such as FMDV.


Figure 1. Antigen-specific humoral immune responses. Anti-FMDV peptide serum IgG (A) and mucosal IgA (B) antibody responses, as measured by ELISA (one animal from the CT group died before completion of the study and was not included in any analyses). IgG concentrations were interpolated from a standard curve and IgA concentrations are expressed as a geometric mean titer (endpoint titer determined to have an absorbance two times higher than background). (C) Virus neutralizing titers as measured by plaque reduction assay (CT was excluded from the analysis as statistics were impeded by the dramatically skewed data distribution of this group (only one animal in this group had measurable VNT, but it was the highest in the study at 1:130)). Data represent the mean response, error bars indicate 1 standard deviation from the mean. Horizontal bars indicate pairwise comparisons between groups with significance denoted by (*) \( p<0.05 \), (**) \( p<0.01 \), (***) \( p<0.001 \).