Evaluation of the adjuvant effect of *Escherichia coli* heat-labile enterotoxin mutant (LTK63) on the systemic immune responses to intranasally co-administered measles virus nucleoprotein. Part I: Antibody responses

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ABSTRACT

The adjuvanticity and immunogenicity of the heat-labile enterotoxin (LT) of *Escherichia coli* and of its non-toxic mutant, LTK63, was evaluated after intranasal administration of CBA mice with recombinant measles virus nucleoprotein (rMVNP) with or without LT or LTK63. Both LT and LTK63 were shown to be highly immunogenic with higher responses observed 4 weeks after the booster immunization. Although the nucleoprotein was immunogenic on its own, mice immunized with the nucleoprotein plus wild type LT produced significantly high antibody responses (p<0.01). Mice that received the rMVNP with LTK63 also generated strong antibody responses to rMVNP. These antibodies were also significantly higher than those of rMVNP alone (p<0.05). No significant differences were observed between groups of mice immunized intranasally with rMVNP plus LT or LTK63 (p>0.05). Data on IgG antibody isotype profiles showed that IgG 1 and IgG 2a were predominant in mice immunized with rMVNP + LT or LTK63 whereas IgG 1 predominated when rMVNP was given on its own implying that LT and LTK63 induce both Th1 and Th2-type immune responses. These results highlight the great potential of this non-toxic mutant of LT as a safe vaccine adjuvant.


INTRODUCTION

The intranasal route has been shown to be an effective route for immunization with various antigens. However, in many instances it may be necessary to increase the immunogenicity of vaccine antigens by use of an appropriate adjuvant.

Cholera toxin (CT) produced by *Vibrio cholerae* and the heat-labile (LT) enterotoxin of *Escherichia coli* have been shown to be potent mucosal immunogens and exert mucosal adjuvanticity to linked or co-administered antigens. These enterotoxins consist of six covalently linked polypeptide chains, comprising of a single A-subunit with NAD-glycohydrolase and ADP-ribosyltransferase activities responsible for activating adenylcyclase in target eucaryotic cells, and five B-subunits that bind the holotoxin to GM1-ganglioside receptors.

The adjuvanticity of these proteins has been a subject of intense investigation but their toxicity precludes their exploitation in vaccines. It is the A-subunit that is toxic. This toxic subunit is responsible for ADP-ribosylation of the GTP binding protein which leads to activation of the adenylcyclase system, persistent cAMP production, and ultimate loss of electrolytes and water from enterocytes with concomitant diarrhea.

One approach being used to resolve the toxicity of CT is the use of the non-toxic B-subunit instead. Apart from being non-toxic, CT-B stimulates good specific immunity when given orally, which has raised hopes for its use as a vaccine adjuvant instead of the holotoxin. In an attempt to overcome the problem of toxicity of LTs and to obtain powerful and safe mucosal adjuvants, a series of mutants of LT have been constructed by site directed mutagenesis, while taking advantage of the known tridimensional structure of LT. This is by introducing single substitutions of the conserved amino acids in the active site of the LT. The results of these manipulations are that LT mutants (such as LTK7 and LTK63) devoid of enzymatic activity have been got. These mutants have been shown to be effective adjuvants for the induction of strong immune responses to a variety of antigens administered mucosally. This includes both humoral and cell-mediated immune responses. However, though the LTK63 mutant was shown to exert a strong adjuvant effect, the use of wild type LT toxin was shown to be a more potent adjuvant for the *in vivo* induction of CTL responses to intranasally co-administered synthetic peptides. This has led to the suggestion that ADP-ribosyltransferase activity may be contributing to the adjuvant activity of the wild type LT toxin.

In this paper, we have critically evaluated the adjuvanticity of the mutant of *Escherichia coli* heat-labile enterotoxin (LTK63), on the humoral immune responses to intranasally co-administered recombinant measles virus nucleoprotein.
Analysis of the isotype profile of antibody responses to rMVNP
The determination of the specific isotypes revealed that, rMVNP when given alone, mainly elicits IgG 1 antibody responses, with low levels of IgG 2a antibodies and hardly any IgG 2b or IgG 3 (figure 3A). However, when the rMVNP was co-administered with LT or LTK63, both IgG 1 and IgG 2a isotypes predominated the IgG response with a little IgG 2b and hardly any IgG 3 (figure 3B and C). Similarly, IgG 1 and IgG 2a predominated IgG response to LT or LTK63 toxins, although, there were some low levels of IgG 2b antibodies. No IgG 3 antibodies were detected (figure 4A, 4B).
Figure 3 IgG isotypes following intranasal immunization with rMVNP alone (A), rMVNP + LT (B), rMVNP + LTK63 (C). Specific isotype responses were determined by ELISA after adding respective pooled group sera (collected on day 21 post immunization) in triplicate to MVNP coated ELISA plates. Serum from group A CBA mice was used at a dilution of 1:50, while that for group B and C CBA mice at 1:500. The OD values represent the mean of triplicate readings.

Figure 4 IgG isotypes after intranasal immunisation with LT or LTK63. Isotypes were determined by ELISA. ELISA plates were coated with GM, followed by LT or LTK63. Respective pooled group sera from CBA mice (collected on day 21-post immunization), was added in triplicate wells. OD values are the mean of triplicate readings.

**Antibody responses to rMNP after subcutaneous immunization**

**Serum antibody responses to rMVNP after subcutaneous administration in Freund's adjuvant (FA)**

To test the immunogenicity of the rMVNP when given subcutaneously, a dose of 30 μg of rMVNP in FA, per mouse, was administered subcutaneously into a group of 6 mice on day 0. Serum antibody responses were monitored by ELISA on a weekly basis. As shown in figure 5, mice showed very high anti-MVNP antibodies in sera by day 21 and thereafter, high levels were sustained upto day 42 post immunization.

Figure 5 Kinetics of the serum antibody response from CBA mice immunized subcutaneously with 30 μg of rMVNP in FA. Immune responses were measured using ELISA. Antibody responses are expressed as mean ± S.D. of titres of serum from 6 mice.
Analysis of IgG isotypes after subcutaneous immunization of CBA mice with rMVNP in Freund's adjuvant

Serum samples analysed for their isotype profile showed the same levels of both IgG 1 and IgG 2a antibody responses (figure 6) and weak IgG 2b antibody levels. No IgG 3 antibodies were detected.

![Graph showing IgG isotypes after subcutaneous immunization of CBA mice with rMVNP in Freund's adjuvant. Specific isotype response was determined by ELISA after adding respective pooled group sera (1:1000 dilution) from group D CBA mice in triplicate to MVNP coated ELISA plates. The OD values represent the mean of triplicate readings for a given isotype ± S.D.](image)

**Graph 6** IgG isotypes after subcutaneous immunization of CBA mice with rMVNP in Freund's adjuvant. Specific isotype response was determined by ELISA after adding respective pooled group sera (1:1000 dilution) from group D CBA mice in triplicate to MVNP coated ELISA plates. The OD values represent the mean of triplicate readings for a given isotype ± S.D.

**DISCUSSION**

The nasal mucosa is the first site of contact with inhaled antigens. The development of vaccines administered via the nasal route offers several advantages for vaccination such as i) a lower dose of antigens is required as the nasal cavity contains less proteolytic activity compared to the intestinal tract, ii) induction of both mucosal and systemic immune responses, and iii) has potential of immunization of large population groups. Because of the easier accessibility of the nasal cavity, this route is under extensive investigation as a potential alternative to parenteral route. However, in many situations it may be necessary to increase the immunogenicity of vaccine antigens by use of an appropriate adjuvant. The LT and CT have been shown to be highly immunogenic and to be the most potent mucosal adjuvants, however, their toxicity precludes their use in vaccines. The construction of non-toxic mutants of CT and LT has been shown to be highly immunogenic as compared to LT. A non-toxic mutant of CT, LTK63, was found to have similar immunogenic potential as LT as adjuvant suggested that a Th2 type T-cell help was induced, while the dominance of IgG 1 and IgG 2a in mice immunized with LT suggested a skew towards a mixed type of response with participation of both Th1 and Th2 cells. The results about isotype analysis in this present study seem to concur with these previous observations. The specific antibodies were primarily IgG 1 when rMVNP was given alone. However, when the rMVNP was given with LT or LTK63, both IgG 1 and IgG 2a isotypes predominated the IgG response. Similarly, IgG 1 and IgG 2a predominated the IgG response. Similarly, IgG 1 and IgG 2a predominated the IgG response when the rMVNP was given alone, it mainly stimulates a Th2 type response whereas when it is given intranasally with LT or LTK63, a balanced immune response of both Th1 and Th2 is obtained.

In this present study, not only was LTK63 used as a mucosal adjuvant, but also an immunogen. When compared with LT toxin, LTK63 mutant was found to have similar immunogenic potential as LT, after intranasal immunization (p<0.05). These findings were in disagreement to previous studies where LT toxin was found to be more immunogenic as compared to LTK63. But the results concur with those reported by other workers who recently described LT mutants completely lacking ADP-ribosyltransferase activity, which were as immunogenic as wild type LT. Although there are contrasting reports, the immunogenicity of these molecules has been suggested to correlate with their adjuvanticity. These findings that LT and LTK63 can enhance immune responses to rMVNP after intranasal immunization represent a significant advance for mucosal vaccine development. These molecules have been said to enhance absorption of antigens by the epithelia and have also been found to abrogate mucosal tolerance. These attributes together with the advantage of the common mucosal immune tractation of rMVNP plus LTK63 were also similar to those when the nucleoprotein was given subcutaneously in Freund's adjuvant (figure 1 and 5). These findings extend the previous studies that have highlighted the potential of LTK63 mutant as an adjuvant for induction of mucosal as well as systemic immune responses to a variety of antigens. Nevertheless, the exact mechanism(s) for the adjuvant effect of LTK63 mutant is unknown. Studies have suggested that the adjuvant effect of CT and LT is linked to their ADP-ribosyltransferase activity. Thus this data seems to cast doubt on the existing dogma based on the idea that ADP-ribosyltransferase activity is necessary for adjuvanticity of LT. It has been suggested that ADP-ribosyltransferase activity may be more important for adjuvanticity of CT than LT.

Although the mechanism by which LTK63 may potentiate immune responses to the nucleoprotein is unknown, previous studies have suggested that CT and LT may potentiate immune responses to bystander proteins via different mechanisms. This theory is supported by the fact that the wild type CT and LT induce measurably different immune responses in vivo. Wild type CT and LT induced different IgG isotype antibody profiles in the serum to OVA. The predominance of IgG 1 antibodies to OVA in mice immunized intranasally with wild type LT as adjuvant suggested that a Th2 type T-cell help was induced, while the dominance of IgG 1 and IgG 2a in mice immunized with LT suggested a skew towards a mixed type of response with participation of both Th1 and Th2 cells. The results about isotype analysis in this present study seem to concur with these previous observations. The specific antibodies were primarily IgG 1 when rMVNP was given alone. However, when the rMVNP was given with LT or LTK63, both IgG 1 and IgG 2a isotypes predominated the IgG response. Similarly, IgG 1 and IgG 2a predominated the IgG response when the rMVNP was given alone, it mainly stimulates a Th2 type response whereas when it is given intranasally with LT or LTK63, a balanced immune response of both Th1 and Th2 is obtained.
system, make the intranasal immunization highly attractive route for vaccination.

These findings have shown that the non-toxic mutant of LT (LTK63) is an effective adjuvant for the induction of nucleoprotein specific immune responses. Its adjuvant effect has proved to be as high as that of wild type LT. LTK63 should therefore be considered as a candidate non-toxic adjuvant for mucosally administered vaccines.

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REFERENCES