Immunology 1998 **94** 424–430

Role of G_{M1} binding in the mucosal immunogenicity and adjuvant activity of the *Escherichia coli* heat-labile enterotoxin and its B subunit

L. DE HAAN,* W. R. VERWEIJ,* I. K. FEIL,†‡ M. HOLTROP,* W. G. J. HOL,†‡§¶ E. AGSTERIBBE* & J. WILSCHUT*

**Department of Physiological Chemistry, Groningen Utrecht Institute for Drug Exploration (GUIDE), University of Groningen, Groningen, the Netherlands, †Howard Hughes Medical Institute and Departments of ‡Biological Structure, §Pharmacology, and ¶Biochemistry, University of Washington, School of Medicine, Seattle, WA, USA*

SUMMARY

Escherichia coli (*E. coli*) heat-labile toxin (LT) is a potent mucosal immunogen and immunoadjuvant towards co-administered antigens. LT is composed of one copy of the A subunit, which has ADP-ribosylation activity, and a homopentamer of B subunits, which has affinity for the toxin receptor, the ganglioside G_{M1}. Both the ADP-ribosylation activity of LTA and G_{M1} binding of LTB have been proposed to be involved in immune stimulation. We investigated the roles of these activities in the immunogenicity of recombinant LT or LTB upon intranasal immunization of mice using LT/LTB mutants, lacking either ADP-ribosylation activity, G_{M1}-binding affinity, or both. Likewise, the adjuvant properties of these LT/LTB variants towards influenza virus subunit antigen were investigated. With respect to the immunogenicity of LT and LTB, we found that G_{M1}-binding activity is essential for effective induction of anti-LTB antibodies. On the other hand, an LT mutant lacking ADP-ribosylation activity retained the immunogenic properties of the native toxin, indicating that ADP ribosylation is not critically involved. Whereas adjuvanticity of LTB was found to be directly related to G_{M1}-binding activity, adjuvanticity of LT was found to be independent of G_{M1}-binding affinity. Moreover, a mutant lacking both G_{M1}-binding and ADP-ribosylation activity, also retained adjuvanticity. These results demonstrate that neither ADP-ribosylation activity nor G_{M1} binding are essential for adjuvanticity of LT, and suggest an ADP-ribosylation-independent adjuvant effect of the A subunit.

INTRODUCTION

The *Escherichia coli* (*E. coli*) heat-labile toxin (LT) and *Vibrio cholerae* cholera toxin (CT) are exceptionally potent mucosal immunogens and immunoadjuvants.[1–5] In addition, LT and CT have been found to abrogate tolerance towards co-administered antigens.[1,3] Both are heterohexameric proteins composed of one copy of the A subunit, which has ADP-ribosylation activity, and five copies of the B subunit. The B-pentamer has high affinity for the toxin receptor, ganglioside G_{M1}. Delivery of the A subunit to the cytosol of the target cell results in persistent synthesis of cAMP.[6,7]

Even though the role of the individual subunits of LT and CT in the toxic mechanism are well defined, their role in the immunogenicity of the molecules and their adjuvanticity towards admixed non-related antigens is not clear. Using LT mutants, which lack ADP-ribosylation activity, we and other investigators have shown that the enzymatic activity of LTA does not play a major role in the immunogenicity of LT.[8–11] On the other hand, recombinant LTB alone is clearly less immunogenic than LT mutants which lack ADP-ribosylation activity,[12] suggesting that the presence of LTA, but not necessarily its enzymatic activity, does contribute to the antitoxin antibody response. The role of the A subunit in adjuvanticity is even less clear. Several studies have shown that recombinant LTB and CTB lack the capacity to stimulate systemic and mucosal antibody responses towards co-administered antigens.[13–18] Furthermore, using an LT mutant lacking ADP-ribosylation activity, Lycke *et al.*[16] showed that the adjuvant effect of LT and CT is directly linked to the enzymatic activity of LTA. By contrast, several other investigators have reported on LT mutants, which lacked ADP-ribosylation activity, but yet retained the adjuvant properties of wild-type LT.[9–11,17–19] Moreover, recently we and others demonstrated that recombinant LTB and CTB also have potent adjuvant activity towards intranasally administered antigens.[19–22]

In general, G_{M1} binding is considered to be essential for immune stimulation by LT and CT. The aim of this study was to investigate the role of G_{M1}-binding activity in the

Received 10 December 1997; revised 2 February 1998; accepted 2 February 1998

Correspondence: Dr J. Wischut, Department of Physiological Chemistry, Groningen Utrecht Institute for Drug Exploration (GUIDE), University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands.

immunogenicity and adjuvant activity of LT and LTB. By means of site-specific mutagenesis, we generated LT and LTB mutants that lack affinity for G_{M1}. Likewise, a double mutant was constructed, which lacks both G_{M1}-binding affinity and ADP-ribosylation activity. These mutants were investigated for their immunogenicity and adjuvant activity towards co-administered influenza HA upon intranasal (i.n.) administration to mice. The responses were compared with those obtained after (co-)immunization with LT, recombinant LTB, and an LT mutant devoid of ADP-ribosylation activity. It is shown that G_{M1} binding is required for the potent immunogenicity of LT and LTB upon i.n. administration. By contrast, adjuvanticity of LT, but not LTB, was found to be largely independent of G_{M1} binding. Finally, it is shown that ADP-ribosylation activity is not required for adjuvanticity, neither in the presence nor in the absence of G_{M1} binding.

MATERIALS AND METHODS

Production and characterization of recombinant LTB, LT and mutants

Recombinant LTB, LT and LT mutant LT-E112K (LTA: Glu112→Lys) were cloned, expressed, and purified as described previously.[12,23] The mutants LTB-G33D and LT-G33D, each with a Gly33→Asp substitution in the B subunit, were constructed by site-specific mutagenesis, sequenced and subcloned in protein expression vector pROFIT.[23] Mutant LT-E112K/G33D (LTA: Glu112→Lys; LTB: Gly33→Asp) was constructed by substituting the *Eco*RIx*Pst*I restriction fragment of pROFIT-LT-E112K[12] for the *Eco*RIx*Pst*I restriction fragment of pROFIT-LTB-G33D. Recombinant proteins were expressed in *E. coli* MC1061 and purified using immobilized D-galactose column chromatography.[12,23,24] Protein samples were found to be free of contaminating bacterial endotoxin in a *Limulus* amoebocyte lysate assay. The G_{M1}-binding capacity of recombinant proteins was determined in a G_{M1}-sandwich enzyme-linked immunosorbent assay (ELISA) and in a competitive G_{M1} ELISA, as described previously.[12,23,25] The enzymatic activity of LT and LT mutants was determined in an ADP-ribosylation assay using diethylaminobenzylidine-aminoguanidine (DEABAG) as an artificial substrate, as previously described.[26,27] Enzymatic activity is expressed as pmol DEABAG converted per nmol protein.

Immunization and antibody assays

Female BALB/c mice (6–8-weeks old) were used (Harlan, Zeist, The Netherlands). Groups consisted of 4 mice each. Mice were immunized i.n. on days 0, 7 and 14 by application of 10 μl of sample on the external nares. In the immunogenicity study, mice received either 2 μg LTB, 2·9 μg LT (equimolar amounts of LTB), 2 μg LTB-G33D, 2·9 μg LT-G33D or 2·9 μg LT-E112K/G33D. In the adjuvant study, mice were given either of the above in conjunction with 5 μg subunit antigen. A monovalent influenza virus subunit preparation was used, derived from strain B/Harbin/7/94 (a generous gift of Solvay Pharmaceuticals B.V., Weesp, The Netherlands), consisting mainly of viral haemagglutinin (HA). Control mice received either subunit antigen alone or phosphate-buffered saline (PBS). Blood samples were taken from the tail vein on days 6, 13 and 20. On day 28 mice were bled and mucosal lavages of the nasal cavity and urogenital tract were performed as previously described.[12,19,23] Antigen-specific antibodies were detected by ELISA, using horseradish peroxidase-conjugated goat-anti-mouse immunoglobulins (IgG or IgA) (Southern Biotechnology Associates, Birmingham, AL), as previously described.[12,19,23] Antibody titres are expressed as the reciprocal serum or mucosal wash dilution with an A_{492} value $\geqslant 0{\cdot}2$ above background. Antibody titres are expressed as geometric mean titres ± standard error of the mean (GMT ± SEM). Presented data are representative of duplicate independent experiments. Comparisons between experimental groups were made by Student's *t*-test. Statistical significance was considered to be indicated by a P value of $<0{\cdot}05$.

RESULTS

Production and characterization of recombinant LTB, LT and mutants

Recombinant proteins were efficiently expressed from pROFIT plasmid constructs in the periplasm of *E. coli* MC1061. Subsequently, proteins were purified using immobilized-D-galactose column chromatography. LTB-G33D, LTB, LT-G33D, LT-E112K, LT-E112K/G33D, and LT assembled properly (Fig. 1a, see also ref. 12). While LTB monomers were visible when samples were boiled prior to sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE), LTB pentamers remained intact without prior heating of samples (Fig. 1a; LT-E112K not shown, but see ref. 12). A difference in the electrophoretic mobility of the mutant LTB on SDS–PAGE was observed, in particular in case of the B-pentamer. The Gly33→Asp mutation introduces a negative charge, which is responsible for this altered mobility.[28] In a G_{M1}-sandwich ELISA, LTB-G33D, LT-G33D and LT-E112K/G33D were found to bind at least 1000-times less efficiently to G_{M1} than LT, LT-E112K, or LTB (results not shown). In addition, in a competition ELISA, involving the use of biotinylated CTB, LTB-G33D, LT-G33D and LT-E112K/G33D were found to have lost the capacity to compete with CTB for binding to G_{M1} (Fig. 1b). LTB, LT and LT-E112K (LT-E112K not shown; see ref. 12) did compete effectively with CTB, exhibiting equal affinities for G_{M1}.

The enzymatic activities of LT, LT-G33D, LT-E112K, and LT-E112K/G33D were determined in an ADP-ribosylation assay using DEABAG as a substrate.[26,27] Recombinant LTB and LTB-G33D were used as negative controls. Table 1 shows that LT-G33D completely retained the wild-type ADP-ribosylation activity. By contrast, like LTB and LTB-G33D, LT-E112K and LT-E112K/G33D failed to convert significant amounts of DEABAG. We conclude therefore, that LT-E112K lacks ADP-ribosylation activity, and that LT-E112K/G33D lacks both ADP-ribosylation and G_{M1}-binding activity.

Immunogenicity of LTB-G33D, LT-G33D and LT-E112K/G33D: induction of LTB-specific antibodies

We recently described the immunogenic properties of LT, LT-E112K, and LTB upon intranasal administration to mice.[12] It was found that LT-E112K largely retained the immunogenic

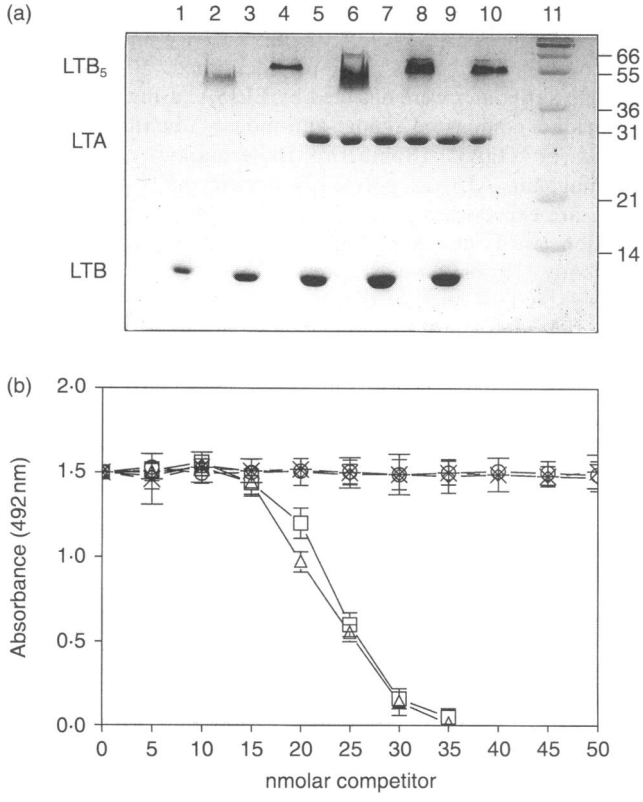

Figure 1. Purification and competitive G_{M1} ELISA of LTB, LT and mutants. (a) SDS–PAGE analysis of LTB, LT and mutants. Lanes 1, 3, 5, 7 and 9, boiled toxin; lanes 2, 4, 6, 8 and 10, unboiled toxin; lanes 1 and 2, LTB; lanes 3 and 4, LTB-G33D; lanes 5 and 6, LT; lanes 7 and 8, LT-G33D; lanes 9 and 10, LT-E112K/G33D; lane 11, molecular weight marker. Molecular weights (in thousands) and LTA, LTB pentamers, and LTB monomers are indicated. (b) Competitive G_{M1} ELISA. Equimolar concentrations of LTB (□), LTB-G33D (○), LT (△), LT-G33D (◇), and LT-E112K/G33D (*) were serially diluted and then mixed with a fixed amount of biotinylated CTB and incubated on a G_{M1}-coated ELISA plate. Values are given as mean ± error of the mean.

Table 1. Comparative ADP-ribosylation activities of wild-type and mutant toxins

Toxin	ADP-ribosylation activity (pmol DEABAG/nmol protein)
LTB	0·68 ± 0·2
LTB-G33D	0·81 ± 0·1
LT	15·9 ± 1·2
LT-G33D	16·8 ± 1·6
LT-E112K	0·54 ± 0·1
LT-E112K/G33D	0·72 ± 0·1

properties of the native toxin. Also LTB was found to be a potent immunogen, although less efficient than LT and LT-E112K, especially with respect to its capacity to induce secretory IgA (S-IgA) at distant mucosal effector sites.[12,23] Thus, we concluded that the A subunit, but not necessarily its ADP-ribosylation activity, contributes to the immunogenicity of LT. To investigate the role of G_{M1} binding in the immunogenic properties of LT and LTB we then investigated the capacity of LTB-G33D, LT-G33D, LT-E112K/G33D, LTB or LT, to induce LTB-specific serum IgG and mucosal S-IgA antibody responses.

Figure 2(a) shows that LTB-G33D failed to induce a detectable serum IgG response, consistent with a key role of G_{M1} binding in the immunogenicity of LTB. Also, LTB-specific antibody responses induced by both LT-G33D and LT-E112K/G33D were markedly reduced, relative to the corresponding responses induced by LT holotoxin. All mutants failed to induce detectable levels of LTB-specific serum IgA (Fig. 2b). LTB did induce detectable, but low levels of serum IgA, while LT induced high levels of serum IgA.

Analysis of nasal and vaginal lavages of the above mice revealed that LTB-G33D also failed to induce a mucosal S-IgA response (Fig. 3). Immunization with LTB resulted in an S-IgA response in the nasal cavity, but, in agreement with our earlier observations,[12,23] migration of the antibody response to the vagina was not observed. Interestingly, both LT-G33D and LT-E112K/G33D completely failed to induce LTB-specific mucosal S-IgA responses. LT induced brisk LTB-specific S-IgA responses in the nasal cavity as well as in the vagina.

The above results demonstrate that loss of G_{M1}-binding affinity markedly reduces the immunogenicity of both LT and LTB. However, additional loss of enzymatic activity does not

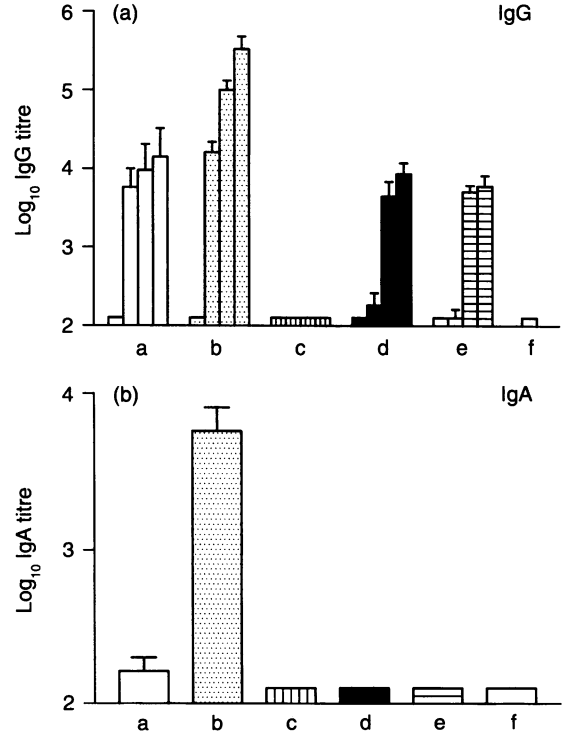

Figure 2. LTB-specific serum antibody responses upon i.n. administration of LTB (a), LT (b), LTB-G33D (c), LT-G33D (d), LT-E112K/G33D (e) and PBS (f). For details, see the Material and Methods section. (A) Serum IgG (determined on days 6, 13 and 20), and, (B) serum IgA (determined on day 28 only). On day 28, differences between wild-type LT-immunized mice and otherwise immunized animals were statistically significant ($P < 0{\cdot}05$; Student's *t*-test).

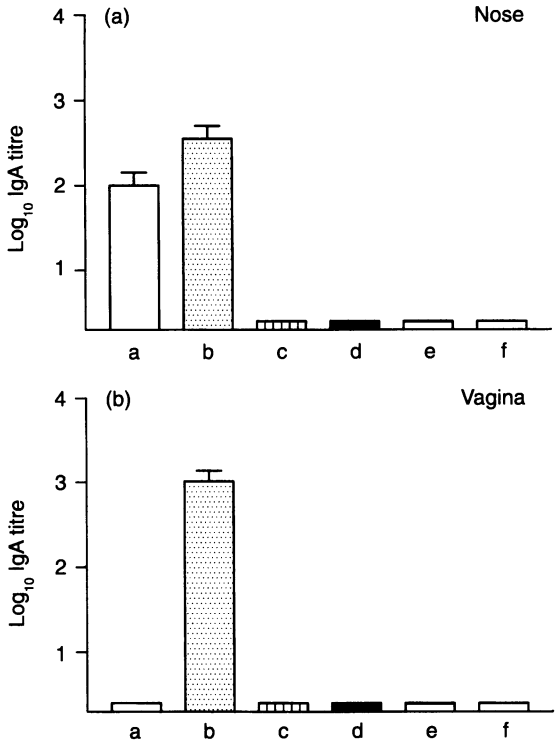

Figure 3. LTB-specific mucosal S-IgA responses upon i.n. administration of LTB (a), LT (b), LTB-G33D (c), LT-G33D (d), LT-E112K/G33D (e) and PBS (f). (A) nasal S-IgA, and (B), vaginal S-IgA. Differences between groups of mice immunized with wild-type LT or otherwise immunized animals were statistically significant ($P<0.05$; Student's *t*-test).

appear to further affect the immunogenicity of LT. We conclude that G_{M1} binding plays an important role in the induction of LTB-specific antibody responses by LT and LTB.

Adjuvant properties of LTB-G33D, LT-G33D, and LT-E112K/G33D towards co-administered influenza subunit antigen

Recently, we demonstrated that not only LT, but surprisingly also recombinant LTB, is a potent mucosal immunoadjuvant towards co-administered antigens.[19,29] Likewise, LT-E112K was found to retain the adjuvant activity.[19,29] We then investigated the role of both G_{M1}-binding and ADP-ribosylation activity in the adjuvant activity of LTB and LT, by immunizing mice as above with influenza subunit antigen alone or in conjunction with either LTB, LT, LT-E112K, LTB-G33D, LT-G33D or LT-E112K/G33D.

Intranasal administration of subunit antigen alone resulted in a poor serum IgG response (Fig. 4). LT acted as a potent adjuvant towards subunit antigen, inducing a brisk serum IgG response. Surprisingly, not only LT-G33D but also LT-E112K/G33D strongly stimulated the IgG response against subunit antigen, indicating that neither G_{M1} binding nor ADP-ribosylation activity are essential for adjuvanticity of LT. In agreement with our earlier observations,[19] the observed IgG titres upon immunization with LTB and LT-E112K were comparable to those obtained with LT. No subunit antigen-specific IgA was detected in serum, using either adjuvant. In

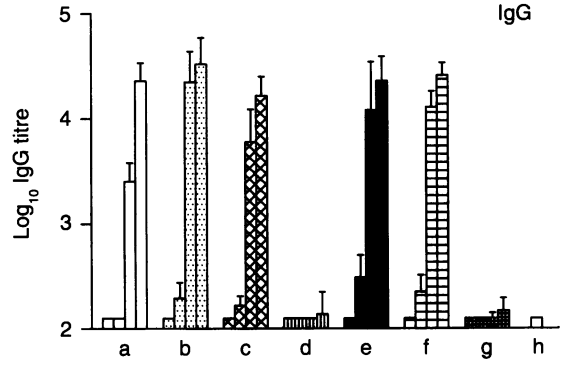

Figure 4. Subunit antigen-specific serum IgG responses upon i.n. immunization with influenza subunit antigen in conjunction with either LTB (a), LT (b), LT-E112K (c), LTB-G33D (d), LT-G33D (e) or LT-E112K/G33D (f) (determined on days 6, 13, 20 and 28). Control mice received subunit antigen alone (g), or PBS (h). For details, see the Materials and Methods section. On day 28, differences between groups receiving either LTB, LT, LT-E112K, LT-G33D or LT-E112K/G33D were not significant ($P>0.05$; Student's *t*-test).

contrast to LT-G33D, LTB-G33D failed to stimulate subunit antigen-specific responses.

Figure 5 shows that i.n. immunization with subunit antigen alone did not result in detectable S-IgA antibody levels, neither in the nasal cavity nor in the vagina. On the other hand, both

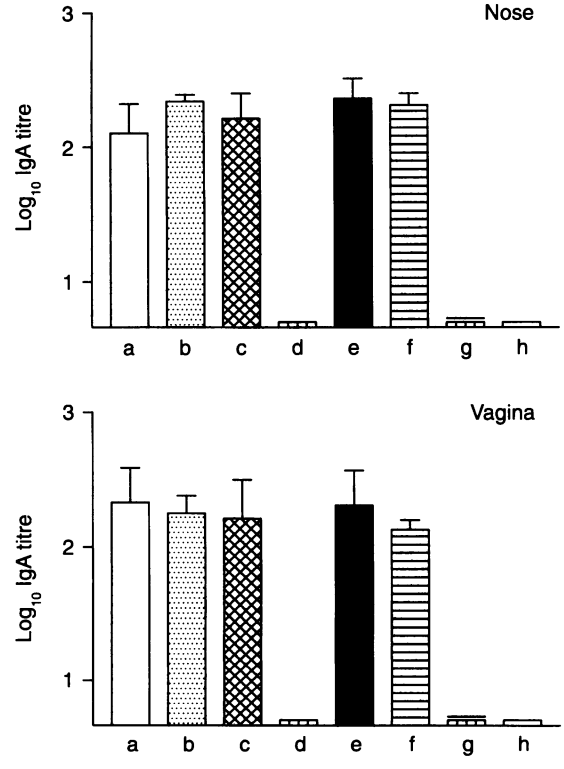

Figure 5. Subunit antigen-specific mucosal S-IgA responses upon i.n. immunization with influenza subunit antigen in conjunction with either LTB (a), LT (b), LT-E112K (c), LTB-G33D (d), LT-G33D (e) or LT-E112K/G33D (f). Control mice received subunit antigen alone (g), or PBS (h). (A) nasal S-IgA, (B) vaginal S-IgA. Differences between the groups receiving either LTB, LT, LT-E112K, LT-G33D or LT-E112K/G33D were not significant ($P>0.05$; Student's *t*-test).

LT-G33D and LT-E112K/G33D completely retained the capacity of LTB, LT-E112K and LT to induce high subunit antigen-specific S-IgA titres in the nose as well as in the vagina. Since no subunit-antigen-specific IgA was detected in serum, we conclude that the IgA found in mucosal lavage fluids was derived from local production, rather than from transudation from serum. LTB-G33D failed to stimulate subunit antigen-specific S-IgA responses to any detectable extent. Thus, induction of influenza subunit antigen-specific serum and mucosal antibodies by LT appears to be largely independent of its affinity for G_{M1}, and, in addition, does not require the ADP-ribosylation activity of the LTA chain.

DISCUSSION

This study demonstrates that G_{M1}-binding affinity is not essential for the adjuvanticity of LT towards i.n. co-administered influenza subunit antigen. It is shown that an LT mutant, which lacks G_{M1}-binding affinity, strongly stimulates subunit antigen-specific systemic IgG as well as mucosal S-IgA responses. Importantly, this immune stimulation does not appear to require the ADP-ribosylation activity of LTA, since a mutant that lacks both ADP-ribosylation activity and G_{M1}-binding also retains adjuvant activity. On the other hand, for induction of LTB-specific antibody responses by LT and LTB, G_{M1} binding is of crucial importance. Also, the adjuvanticity of LTB is completely dependent on its affinity for G_{M1}. Together, these results indicate that LTA is involved in the adjuvanticity of LT in a manner independent of the G_{M1}-binding or ADP-ribosylation activities of the molecule.

Jobling *et al.*[30] were the first to thoroughly investigate the effect of point mutations on the G_{M1}-binding properties of CTB. It was found that substitution of glycine at position 33 with aspartic acid, glutamic acid or leucine, completely abolished G_{M1} binding of CTB.[30] Accordingly, and in agreement with Nashar *et al.*[28] and Guidry *et al.*,[31] our present results demonstrate that the G33D mutation abolishes G_{M1} binding of LT or LTB. In the ADP-ribosylation assay, LT-G33D completely retained the capacity of wild-type LT to convert the DEABAG substrate. By contrast, with LT-E112K and LT-E112K/G33D no conversion of the substrate was observed. This is in agreement with our earlier observations,[12,19,29] and observations of others,[16] showing that the E112K substitution abolishes the capacity of LT to ADP-ribosylate artificial substrates or to enhance intracellular cAMP levels.

Our results demonstrate that the immunogenic and adjuvant properties of LTB are dependent on its capacity to bind to G_{M1}, since LTB-G33D completely failed to induce LTB and subunit antigen-specific antibody responses. Similarly, Nashar *et al.*[19] found that LTB-G33D is non-immunogenic using the subcutaneous or oral route of immunization. Loss of G_{M1}-binding affinity also markedly reduced the immunogenicity of LT. Serum IgG antibody levels observed with LT were much higher than with LT-G33D, while, in addition, LT, and not LT-G33D, was a potent inducer of serum IgA and mucosal S-IgA. These results are in good agreement with recent findings by Guidry *et al.*,[31] showing that the immunogenicity of LT upon oral administration is also dependent on G_{M1}-binding affinity. Interestingly, the immunogenicities of LT-G33D and LT-E112K/G33D were quite comparable. Thus, we conclude that G_{M1} binding is essential for the immunogenicity of LT/LTB, and that, consistent with earlier observations,[8–12,17,18] ADP-ribosylation activity does not play a major role in the antitoxin-antibody response.

The most important result of this paper is the finding that G_{M1} binding does not appear to be essential for the adjuvant activity of LT; LT and LT-G33D mediated comparable antibody responses against co-administered influenza subunit antigen. Since LT-G33D is only weakly immunogenic, these results demonstrate that the immunogenicity of LT is not necessarily linked with adjuvanticity. Our current observations are in contrast to recent findings by Guidry *et al.*[31] who found that LT-G33D lacks adjuvanticity towards orally administered ovalbumin (OVA). This apparent discrepancy may be explained by the difference in route of immunization, intranasal in our study, and oral in the studies of Guidry *et al.*[31] Also, the type of antigen used, and the affinity of the antigen for mucosal surfaces may have played a role in the observed adjuvant effect. In contrast to influenza subunit antigen, OVA, which was used in the studies of Guidry *et al.*,[31] is a poor immunogen when given mucosally. However, very recent studies in our laboratory have shown that LT-G33D also retains adjuvanticity towards keyhole limpet haemocyanin (KLH), which does not have specific binding properties and is also a poor mucosal immunogen (De Haan *et al.*, in press). In addition, it should be noted that in the studies of Guidry *et al.*[31] at least partial adjuvanticity of LT-G33D was observed, indicating that indeed the route of immunization may be crucial. We cannot exclude the possibility that binding of LT-G33D to a different receptor, such as non-ganglioside glycolipids and glycosylated proteins,[32,33] has played a role in its adjuvant properties. However, the results obtained with LTB-G33D suggest that G_{M1} binding is essential for LTB-mediated immune stimulation. Together, these results suggest that two separate adjuvant mechanisms, mediated by either the A or the B subunit, are involved in the overall adjuvant effect of LT. Indeed recently, we observed that the recombinant LTA subunit may also, by itself, act as a powerful adjuvant towards non-related antigens (De Haan *et al.* submitted).

There is an ongoing discussion on the role of ADP-ribosylation activity in the immunogenic and adjuvant properties of LT and CT. This has been investigated using either LTB/CTB alone, or LT/CT mutants, which lack ADP-ribosylation activity. While Lycke *et al.*[16] observed that neither recombinant CTB nor LT-E112K retained adjuvant activity, several studies have shown that non-toxic LT/CT mutants, including LT-E112K and CT-E112K, retain adjuvant activity.[9–13] Moreover, there is increasing evidence that recombinant LTB and CT also have adjuvant activity comparable to that of the native toxin.[19–22] It should be noted, however, that so far, adjuvanticity of non-toxic LT mutants and recombinant LTB/CTB has only been observed when the nasal or subcutaneous route of administration was used, suggesting that the routing of antigen and adjuvant is indeed important. With respect to ADP-ribosylation activity, our current results, obtained with the LT-E112K/G33D double mutant, show that also in the absence of G_{M1} binding, the ADP-ribosylation activity of LTA is not required for the adjuvanticity of LT. Remarkably, LT-E112K/G33D stimulated subunit antigen-specific responses to the same extent as wild-type LT. Thus,

we conclude that neither ADP-ribosylation activity LTA, nor G_{M1}-binding activity of LTB, is essential for adjuvanticity of LT.

Although we have observed that recombinant LTB, LT-G33D and LT-E112K/G33D retain adjuvant activity, to an extent completely comparable to that of wild-type LT, it is possible that there are qualitative differences between these responses. For instance, the mode of induction, e.g. G_{M1}-dependent or -independent, may influence the T-helper response induced, and thereby selectively induce different antibody-subtype profiles. At any rate, the present demonstration of G_{M1} binding and ADP-ribosylation-independent adjuvanticity of LT, has important implications for the development of locally administered vaccines based on the use of LT as a mucosal adjuvant.

ACKNOWLEDGMENTS

This study was supported by the Netherlands Organization for Scientific Research (NWO) under the auspices of the Council for Medical Research (GB-MW) (Research Grant 900-515-048, supporting W.R.V.). We wish to thank Dr R. Brands and Dr G.J.M. van Scharrenburg (Solvay Pharmaceuticals, Weesp, The Netherlands) for stimulating discussions and ample supplies of influenza subunit material.

REFERENCES

1. Clements J.D., Hartzog N.M. & Lyon F.L. (1988) Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine* **6,** 269.
2. Elson C.O. & Ealding W. (1984) Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J Immunol* **132,** 2736.
3. Elson C.O. & Ealding W. (1984) Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. *J Immunol* **133,** 2892.
4. Lycke N. & Holmgren J. (1986) Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology* **59,** 301.
5. Snider D.P. (1995) The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. *Crit Rev Immunol* **15,** 317.
6. Spangler B.D. (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol Rev* **56,** 622.
7. Sears C.L. & Kaper J.B. (1996) Enteric bacterial toxins: mechanism of action and linkage to intestinal secretion. *Microbiol Rev* **60,** 167.
8. Pizza M., Fontana M.R., Giuliani M.M. *et al.* (1994) A genetically detoxified derivative of heat-labile *Escherichia coli* enterotoxin induces neutralizing antibodies against the A subunit. *J Exp Med* **180,** 2147.
9. Douce G., Turcotte C., Cropley I. *et al.* (1995) Mutants of *Escherichia coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as nontoxic, mucosal adjuvants. *Proc Natl Acad Sci USA* **92,** 1644.
10. Dickinson B.L. & Clements J.D. (1995) Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infect Immun* **63,** 1617.
11. Di Tommaso A., Saletti G., Pizza M. *et al.* (1996) Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. *Infect Immun* **64,** 974.
12. DeHaan L., Verweij W.R., Feil I.K. *et al.* (1996) Mutants of the *Escherichia coli* heat-labile enterotoxin with reduced ADP-ribosylation activity or no activity retain the immunogenic properties of the native holotoxin. *Infect Immun* **64,** 5413.
13. Vadolas J., Davies J.K., Wright P.J. & Strugnell R.A. (1995) Intranasal immunization with liposomes induces strong mucosal immune responses in mice. *Eur J Immunnol* **25,** 969.
14. Tamura S.-I., Yamanaka A., Shimohara M. *et al.* (1994) Synergistic action of cholera toxin B subunit (and *Escherichia coli* heat-labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. *Vaccine* **12,** 419.
15. Tamura S.-I., Asanuma H., Tomita T. *et al.* (1994) *Escherichia coli* heat-labile enterotoxin B subunits supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine. *Vaccine* **12,** 1083.
16. Lycke N., Tsuji T. & Holmgren J. (1992) The adjuvant effect of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. *Eur J Immunol* **22,** 2277.
17. Yamamoto S., Takeda Y., Yamamoto M. *et al.* (1997) Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. *J Exp Med* **185,** 1203.
18. Yamamoto S., Kiyono H., Yamamoto M. *et al.* (1997) A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc Natl Acad Sci USA* **94,** 5267.
19. Verweij W.R., DeHaan L., Holtrop M. *et al.* (1989) Mucosal immunoadjuvant activity of the recombinant *Escherichia coli* heat-labile enterotoxin and its B subunit: Induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. *Vaccine*, in press.
20. Hazama M., Mayumi-Aono A., Miyazaki T., Hinuma S. & Fujisawa Y. (1993) Intranasal immunization against herpes simplex virus by using a recombinant glycoprotein D fused with immunomodulating proteins, the B subunit of *Escherichia coli* heat-labile enterotoxin and interleukin-2. *Immunology* **78,** 643.
21. Tochikubo K., Isaka M., Yasuda Y. *et al.* (1998) Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. *Vaccine* **16,** 150.
22. Wu H.-Y. & Russell M.W. (1998) Induction of mucosal and systemic immune responses by intranasal immunization using recombinant cholera toxin B subunit as an adjuvant. *Vaccine* **16,** 286.
23. DeHaan L., Verweij W.R., Holtrop M., Agsteribbe E. & Wilschut J. (1996) Mucosal immunogenicity of the *Escherichia coli* heat-labile enterotoxin: Role of the A subunit. *Vaccine* **14,** 260.
24. Uesaka Y., Otsuka Y., Lin Z. *et al.* (1994) Simple method of purification of *Escherichia coli* heat-labile enterotoxin and cholera toxin using immobilized galactose. *Microb Pathol* **16,** 71.
25. Dertzbaugh M.T. & Elson C.O. (1993) Reduction in oral immunogenicity of cholera toxin B subunit by N-terminal peptide addition. *Infect Immun* **61,** 384.
26. Feil I.K., Reddy R., DeHaan L. *et al.* (1996) Protein engineering studies of A-chain loop 47 of *E. coli* heat-labile enterotoxin point to a prominent role of this loop for cytotoxicity. *Mol Microbiol* **20,** 823.
27. Soman G., Narayanan J., Martin B.L. & Graves D.J. (1986) Use of substituted benzylidineamino-guanidines in the study of guanidino group specific ADP-ribosyltransferase. *Biochemistry* **25,** 4113.
28. Nashar T.O., Webb H.M., Eaglestone S., Williams N.A. & Hirst T.R. (1996) Potent immunogenicity of the B subunits of *Escherichia coli* heat-labile enterotoxin: Receptor binding is essential and induces differential modulation of lymphocyte subsets. *Proc Natl Acad Sci USA* **93,** 226.
29. De Haan L., Verweij W.R., Holtrop M. *et al.* (1998) Nontoxic variants of the *Escherichia coli* heat-labile enterotoxin as mucosal immunogens and adjuvants. *STP Pharma Sciences* **8,** 75.

30. Jobling M.G. & Holmes R.K. (1991) Analysis of structure and function of the B subunit of cholera toxin by the use of site-directed mutagenesis. *Mol Microbiol* **5,** 1755.
31. Guidry J.J., Cardenas L., Cheng E. & Clements J.D. (1997) Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *Escherichia coli* heat-labile enterotoxin. *Infect Immun* **65,** 4943.
32. Holmgren J., Fredman P., Lindblad M., Svennerholm A.M. & Svennerholm L. (1982) Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin. *Infect Immun* **38,** 424.
33. Teneberg S., Hirst T.R., Angstrom J. & Karlsson K.A. (1994) Comparison of cholera toxin and porcine *Escherichia coli* heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine. *Glycocon J* **11,** 533.