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Intranasal Immunogenicity and Adjuvanticity of Site-Directed Mutant Derivatives of Cholera Toxin

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Genetically modified derivatives of cholera toxin (CT), harboring a single amino acid substitution in and around the NAD binding cleft of the A subunit, were isolated following site-directed mutagenesis of the *ctxA* gene. Two mutants of CT, designated CTS106 (with a proline-to-serine change at position 106) and CTK63 (with a serine-to-lysine change at position 63), were found to have substantially reduced ADP-ribosyltransferase activity and toxicity; CTK63 was completely nontoxic in all assays, whereas CTS106 was 10^4 times less toxic than wild-type CT. The mucosal adjuvanticity and immunogenicity of derivatives of CT were assessed by intranasal immunization of mice, with either ovalbumin or fragment C of tetanus toxin as a bystander antigen. Mice immunized with wild-type CT produced both local (immunoglobulin A in mucosal washes) and systemic immune responses to both CT and bystander antigens. CTS106 showed good local and systemic responses to bystander proteins and to itself. Interestingly, mice immunized with the nontoxic derivative of CT, CTK63, generated weak immune responses to the bystander antigens which were similar to those achieved when CT B subunit was used as an adjuvant. In parallel experiments, an equivalent nontoxic mutant of the *Escherichia coli* heat-labile enterotoxin, LTK63 (with a serine-to-lysine change at position 63), was tested (9). In contrast to CTK63, LTK63 was found to be more immunogenic and a better intranasal adjuvant than recombinant heat-labile enterotoxin B subunit or CTK63. This information, together with data on immunoglobulin subclass responses, suggests that although highly homologous, CT and heat-labile enterotoxin should not be considered biologically identical in terms of their ability to act as intranasal adjuvants.

Cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) from *Escherichia coli* are strong mucosal adjuvants capable of modifying immune responses to mucosally coadministered bystander antigens (2, 10, 15, 18). For example, they can activate the production of secretory and humoral antibody responses to antigens that are normally poor mucosal immunogens, following coadministration to mucosal surfaces. These toxins have consequently been used extensively as adjuvant components of experimental mucosal vaccines, particularly in rodents (4, 16, 22, 28). However, the potent toxicity of these proteins to humans has prevented further exploitation of these adjuvants in the development of new mucosally delivered human vaccines (17).

CT and LT are ADP-ribosylating toxins which have considerable amino acid sequence homology and which are highly related in terms of subunit and functional organization (32). Both toxins are heterohexameric proteins composed of an enzymatically active A subunit and a pentameric B subunit which mediate binding of the holotoxins to receptors at the surface of eucaryotic target cells (31). In an attempt to circumvent the problem of toxicity for vaccine development, the adjuvant activity of the nontoxic B subunits of these toxins has been evaluated (36, 37). However, many of the published reports described experiments in which commercial preparation of CT B subunit (CT-B) were used. These preparations are believed to be potentially contaminated with a small but biologically significant amount of active toxin. Subsequent studies using both recombinant CT-B (rCT-B) and rLT-B, prepared from nontoxic heterologous host microorganisms such as *E. coli*, have suggested that LT-B (2) and CT-B (19) may be poor mucosal adjuvants and that only the addition of native holotoxin can provoke strong bystander responses (38). More recently, CT-B has been described as a transmucosal carrier protein which can be used to induce (or adjuvant) peripheral immunological tolerance to directly conjugated proteins (34).

Purified preparations of both LT and CT have been characterized by use of X-ray crystallography (29, 30). The X-ray structures have been used as a basis for molecular modelling to identify candidate amino acids within the polypeptide chains of the A and B subunits as targets for mutagenesis to map residues essential for different toxin-associated biological activities. A number of genetically modified LT and CT derivatives that are still able to assemble into holotoxin but which exhibit significantly reduced toxicity or ADP-ribosyltransferase activity have been described previously (5, 11, 25). Purification of such proteins allows the direct comparison, in biological assays, of holotoxin derivatives which are structurally conserved but functionally distinct (7, 9). Some LT holotoxin derivatives have been created to investigate the relationship between toxicity associated with ADP-ribosyltransferase activity and mucosal adjuvanticity, and derivatives of these that have modified toxicity on Y1 cells but still retain some mucosal adjuvant activity have been described (6).

Here, we describe how mutants of both LT and CT were evaluated in a murine system for their ability to act as mucosal immunogens or adjuvants for coadministered bystander antigens. In this paper, we first describe experiments using two mutants of CT. One mutant, CTS106 (with a proline-to-serine change at position 106), retains a low level of ADP-ribosyltransferase and toxic activity, while a second mutant, CTK63 (with a serine-to-lysine change at position 63), is devoid of any

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similar detectable activity. We then describe experiments using equivalent derivatives of LT (LTK63 [with a serine-to-lysine change at position 63]) and CT (CTK63) which have been genetically detoxified by substitution of an identical amino acid. These derivatives appear to differ in their ability to act as intranasal (i.n.) immunogens and as i.n. adjuvants to bystander proteins.

MATERIALS AND METHODS

Mice. Female C57/B6 mice aged between 6 and 8 weeks were obtained from Harlan Olac (Bichester, United Kingdom).

Antigens. Wild-type CT, site-directed mutants CTS106 and CTK63, and rCT-B were constructed and purified by M. Fontana (11). Wild-type toxin LT, site-directed mutant LTK63, and rLT-B were made and purified by M. Pizza (26). Ovalbumin (OVA) was purchased from Sigma (Poole, United Kingdom), and fragment C, the nontoxic C-terminal domain from tetanus toxin, was a kind gift from Steve Chatfield (Vaccine Research Unit, Medeva, London, United Kingdom).

Toxicity tests. Toxicity of the mutants was tested in vitro with Y1 cells and in vivo by the rabbit ileal loop test (12). In the Y1 cell assay, twofold dilutions of active toxin (either LT or CT) or derivatives were added to wells containing 5×10^4 cells from a concentration of 80 pg/well, and the morphology of the cells was monitored after 24 and 48 h.

Immunization of mice. The mucosal immunogenicity and adjuvanticity of mutant toxins were tested by immunizing groups of mice i.n. with 1 μg of toxin and 10 μg of bystander antigen. For immunizations, proteins were resuspended in phosphate-buffered saline (pH 7.2; PBS) and delivered in a volume of 30 μl (15 μl per nostril) per mouse. To assess adjuvanticity with OVA as the bystander antigen, mice were immunized with either 10 μg of OVA alone or 10 μg of OVA and 1 μg of toxin derivatives. The animals were immunized on days 1, 21, 35, and 61. Immune responses were monitored in serum samples taken on days 0, 20, 34, 56, and terminally on day 76. Mucosal antibody estimations were made from mucosal lavages taken on day 76. To assess adjuvanticity with fragment C as the bystander antigen, mice were immunized with either 10 μg of fragment C alone or 10 μg of fragment C and 1 μg of toxin derivative. The animals were immunized on days 1 and 21. Immune responses were monitored by using blood samples taken on days 0, 20, and terminally on day 34. Mucosal antibody estimations were made from mucosal lavages taken on day 34.

Measurement of antibodies in mucosal secretions. Nasal and lung lavages were performed on all animals. In brief, animals were culled by cardiac puncture and dissected so that the trachea was exposed. An ultrathin pipette was then inserted into a small nick in the trachea. Lung washes were performed by repeated flushing and aspiration of 1.5 ml of 0.1% bovine serum albumin (BSA), in PBS, into the lungs. Nasal washes were collected by flushing 1 ml of 0.1% BSA in PBS through the nasal cavity.

ELISA. Toxin-specific antibodies were measured with a GM1 capture enzyme-linked immunosorbent assay (ELISA). For estimation of OVA-specific antibodies, plates were coated with 60 μg of OVA per ml. For estimation of fragment C-specific antibodies, plates were coated with 10 μg of tetanus toxoid per ml. In brief, for all ELISAs, 96-well plates were coated with antigen overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween 20 and blocked for 1 h at 37°C with 1% BSA. Sera from individual mice were tested by using fivefold serial dilutions of 1:50. Serum samples were incubated for 2 h at 37°C. The plates were washed as described above and incubated with an appropriate antimouse antibody. Total immunoglobulin was measured with rabbit anti-mouse antibody-horseradish peroxidase (HRP) conjugate (Dako, High Wycombe, United Kingdom) for 2 h 37°C, and bound antibody was visualized by adding *o*-phenylenediamine substrate (OPD; Sigma). Immunoglobulin G (IgG) subclass antibodies were determined with either IgG1, IgG2a, IgG2b, or IgG3 biotinylated antibody (Pharmingen, Cambridge BioScience, Cambridge, United Kingdom). HRP-streptavidin (Dako) was then added to each well (1:1,000), and the wells were incubated for 1 h at 37°C. Bound antibody was visualized with OPD substrate as described above. Quantitation of antibody was estimated by endpoint ELISA. Absorbancies were read at 490 nm, and ELISA titers were determined arbitrarily as the dilution of serum which gave an optical density value of 0.3 above the level measured in preimmune samples. Standardization of the results was made possible by the inclusion on each plate of a standard control serum of known titer.

Estimation of specific IgA in mucosal secretions. Titers of specific IgA were measured as described above except that instead of serum, mucosal lavages from individual mice were added to the wells. Lavages were tested in fivefold dilutions by using undiluted lavage in the top well. After incubation overnight at 4°C, the plates were washed as described above and incubated with α-chain-specific biotin conjugate antibody (Sigma) for 2 h at 37°C. Bound antibody was detected by use of streptavidin-HRP (Dako), followed by OPD substrate as described above. ELISA units were determined arbitrarily as the dilution of lavage which gave an optical density value at 490 nm of 0.2 above that of nonimmunized controls. These results are not expressed in terms of total IgA since after performing several studies, we became aware that the levels of total IgA were consistently higher in the washes from mice immunized with toxin or derivatives as an adjuvant. The increase in the level of IgA in these mice appeared to be an indirect consequence of the mucosal IgA immune response to the toxins. Since calculation of OVA-specific IgA/total IgA ratios could not account for the IgA response to the toxin derivatives, which were alone (especially in the case of CT derivatives) highly variable, such ratios were considered inappropriate.

Statistical analysis. Comparisons of specific antibody responses were made with the Student *t* test, where *P* values of <0.05 were considered to be statistically significant.

RESULTS

i.n. adjuvant activity of mutant CT derivatives. Groups of five C57/B6 mice were immunized i.n. with 10 μg of OVA alone or 10 μg of OVA together with either 1 μg of wild-type CT, CTS106, CTK63, or CT-B. Mice were immunized up to four times, and blood samples were prepared from the different groups after each immunization. As can be seen in Fig. 1a, the adjuvant activity of the mutants of CT exhibited a direct correlation with the level of ADP-ribosyltransferase enzymatic activity (Table 1). Wild-type CT induced the highest anti-OVA immunoglobulin responses in the serum. This immunoglobulin response was detectable after a single i.n. immunization (day 20) and was subsequently boosted following a second i.n. immunization. Mutant CTS106, which retains a low level of ADP-ribosyltransferase activity (Table 1), was a less effective mucosal adjuvant, inducing detectable anti-OVA responses in the serum after only two i.n. doses. After four i.n. doses, all mice in this group showed high levels of anti-OVA in the sera, although this level was still at least five times lower than the titers measured in mice immunized with the native toxin (statistically significant [$P = 0.02$]). Mutant CTK63, a holotoxin derivative with no detectable ADP-ribosyltransferase activity of toxicity, was a poor mucosal adjuvant for OVA, inducing low levels of anti-OVA in the serum. These levels were significantly lower than those induced by CTS106 ($P = 0.001$). After two immunizations, these responses were actually equivalent to those achieved when the B subunit alone was used (data not shown). As the animals received subsequent immunizations, a small difference between the adjuvant activity of CTK63 and rCT-B was detected. However, these differences do not appear to be statistically significant ($P = 0.23$).

A similar pattern of i.n. adjuvant activity was observed when fragment C was used as the bystander antigen. Anti-fragment C serum responses in individual mice are shown in Fig. 1b. All 10 mice immunized with wild-type CT contained high levels of anti-fragment C in their serum after two i.n. doses, whereas mice immunized with CTS106 had an approximate 10-fold reduction in response to fragment C. The nontoxic mutant CTK63 induced an immune response which was approximately 200-fold less than that generated with wild-type CT as the mucosal adjuvant. As observed with OVA, after two i.n. doses, the anti-fragment C response generated by CTK63 was only equivalent to that induced with rCT-B ($P = 0.8$). In contrast, the titer of the anti-fragment C response generated by CTS106 was significantly higher than that induced by CTK63 ($P = 0.03$).

The i.n. adjuvant activity of each CT derivative was monitored by measuring the levels of anti-OVA antibody-specific IgA in nasal and pulmonary washes (Fig. 2). IgA against the bystander antigen was detected only when CT derivatives which retained toxic activity were used as i.n. adjuvants. The highest IgA response to the bystander antigens was observed in lavages from both the nose and lungs of mice immunized with wild-type CT. Mice immunized with CTS106 as the i.n. adjuvant produced OVA-specific IgA antibody, although the level was between 5 and 10 times less than that observed with the native CT. OVA-specific IgA was observed only in one of five

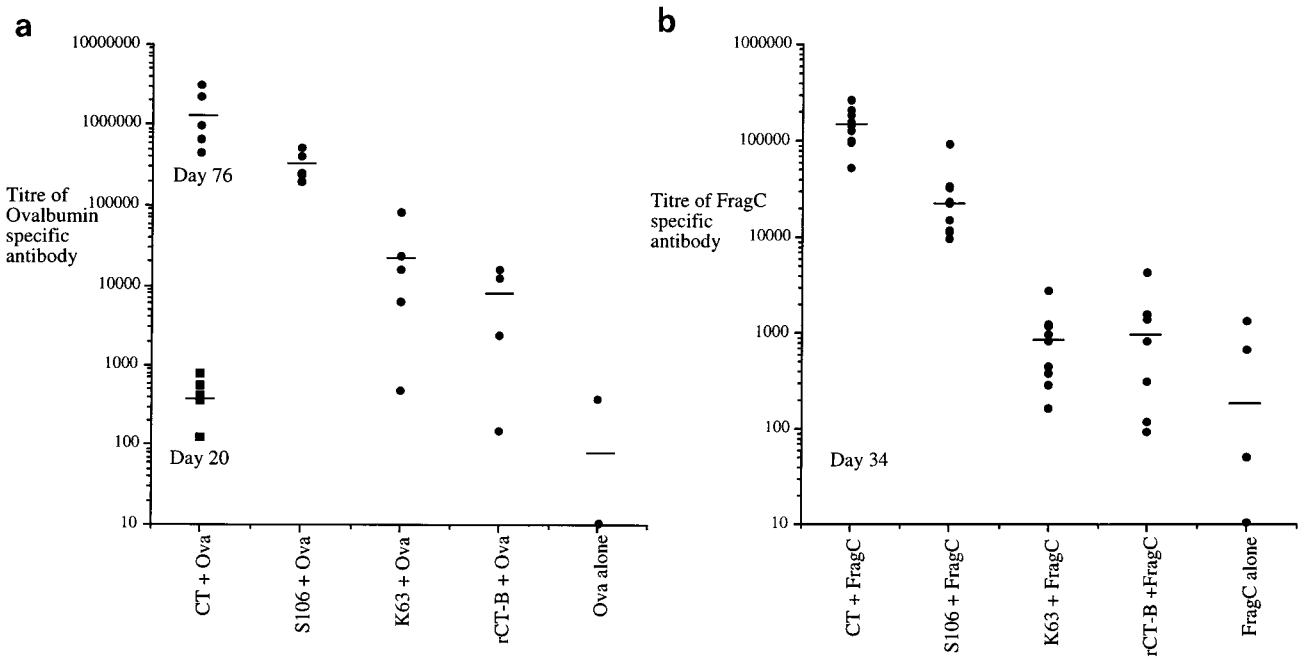


FIG. 1. Immune responses to the bystander antigen generated in the sera of mice immunized i.n. with CT or detoxified derivatives of CT. (a) Individual titers of OVA-specific antibody in the sera of five mice taken after one (■) or four (●) immunizations; (b) individual titers measured on day 34 (after two immunizations) for 10 mice per group immunized i.n. with fragment C (Frag C) alone or fragment C admixed with CT or the detoxified derivatives of CT. The mean titer per group in indicated by a horizontal bar.

lung lavages taken from mice immunized with CTK63 (significantly lower than that observed with CTS106; $P = 0.03$ for both nasal and lung lavages). No IgA response was measured in any of the mice immunized with CT-B.

Antibody responses to CT derivatives following i.n. immunization. Mice immunized i.n. with CT derivatives all possessed detectable levels of anti-CT antibodies in their serum following immunization (Fig. 3a). Interestingly, these results clearly show that the immunogenicities of CTS106 and CTK63 were not equivalent, and both mutants were less immunogenic than wild-type CT. Data from several experiments confirmed that wild-type CT was consistently more immunogenic than CTK63, CTS106, or rCT-B proteins. In general, the immune response to the mutant CTS106 was approximately 10-fold less than that induced by wild-type CT. IgA-specific anti-CT responses were also detected in mucosal washes following i.n. immunization (Fig. 3b and c). In the nasal and lung lavages, the anti-CT IgA titer was again highest in mice immunized with wild-type CT; mutant CTS106 induced intermediate IgA responses, and CTK63 induced a lower response. These data suggests that ADP-ribosyltransferase activity may be important, at least for these particular mutants of CT, not only for inducing immune responses to bystander proteins, particularly mucosal IgA responses, but also for possibly maximizing the antibody immune response to CT. However, other possible explanations exist (see Discussion).

TABLE 1. In vitro and in vivo toxicity of mutants of LT and CT

Protein tested	Amino acid change	Toxicity on Y1 cells	Toxicity in rabbit ileal loops
Native CT		Toxic at 5 pg	Toxic at 50 ng
CTS106	Pro-106 to Ser	Toxic at 600 ng	Toxic at 100 μg
CTK63	Ser-63 to Lys	Nontoxic at 70 μg[a]	Nontoxic at 1 mg[a]
Native LT		Toxic at 2.5 pg	Toxic at 10 ng
LTK63	Ser-63 to Lys	Nontoxic at 40 μg[a]	Nontoxic at 1 mg[a]

[a] Highest concentration tested.

Comparison of the i.n. immunogenicity and adjuvanticity of CTK63 and LTK63. The observation described above that enzymatic activity appears to correlate with adjuvant activity of CT differs from our previous observation that nontoxic derivatives of LT retain some adjuvant activity (9). The nontoxic LT mutant, LTK63, harboring an amino acid substitution equivalent to that in CTK63, has been described previously (7, 26). LTK63 is nontoxic in several toxicity assays. To make a side-by-side comparison of the i.n. adjuvanticity and immunogenicity of equivalent nontoxic mutants of LT and CT, mice were immunized i.n. with either OVA alone or OVA with wild-type CT, CTK63, wild-type LT, or LTK63.

In terms of immunogenicity, CTK63 induced significantly lower levels of either serum or local antibody than the native protein even after several immunizations. In contrast, LTK63 induced antibody responses in the serum which were equivalent to those induced by wild-type LT. High levels of LT-specific IgA were also detected in the nasal and lung lavages of mice immunized with LTK63 (data not shown). These mutants also displayed sharply contrasting levels of i.n. adjuvanticity. Figure 4 shows the serum response to OVA in mice independently immunized i.n. with wild-type LT, wild-type CT, LTK63, or CTK63 and the rLT-B or rCT-B. Wild-type LT and CT were the most efficient i.n. adjuvants, with anti-OVA responses detectable after a single i.n. dose. However, the immune response generated after two i.n. doses in the presence of LTK63 was only five times less than that of wild-type LT (Fig. 4b). In contrast, the immune response to bystander generated by CTK63 (Fig. 4a) was 200-fold less than that generated by the wild-type CT after four immunizations. Although both LTK63

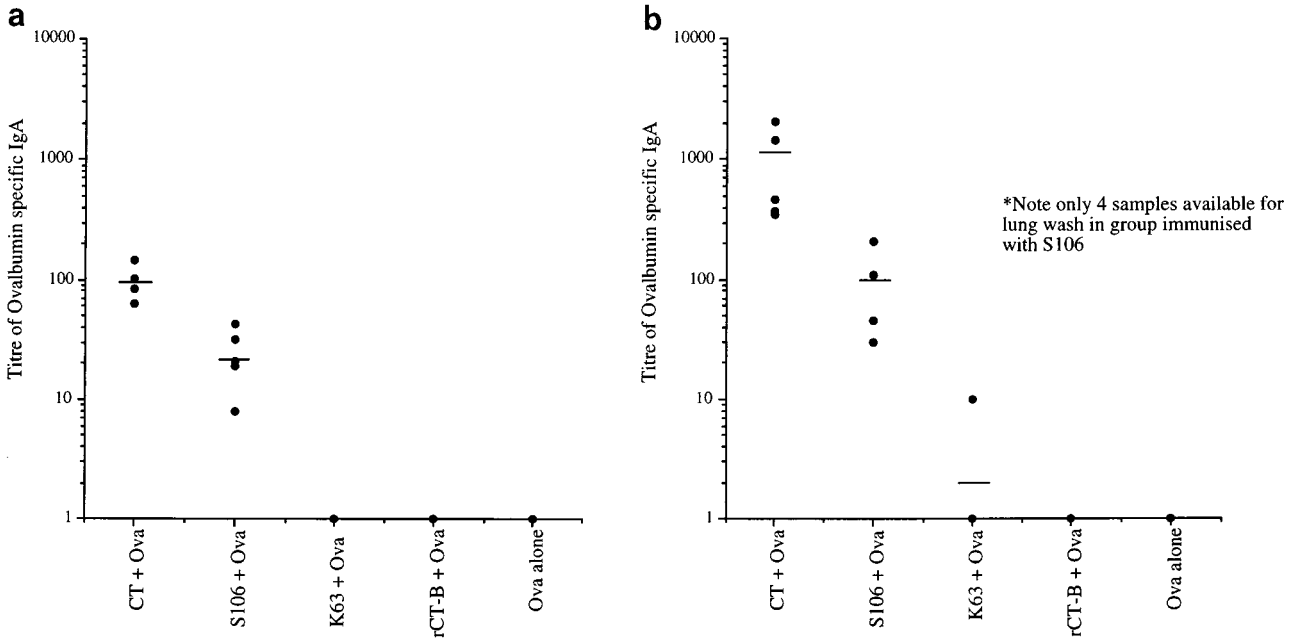


FIG. 2. Mucosal IgA response in the nasal and pulmonary lavages taken from mice after four i.n. immunizations with OVA alone or OVA admixed with CT or the detoxified derivatives of CT. (a) Titers of OVA-specific IgA in the nasal lavages of individual mice; (b) titers of OVA-specific IgA in pulmonary lavages of individual mice.

and CTK63 generated immune responses which were significantly less than that of the native toxin ($P = 0.012$ and 0.02 respectively), the immune response generated by LTK63 was significantly higher than that induced by CTK63 ($P = 0.01$). Interestingly, rLT-B appears to be more effective at stimulating serum antibody responses to OVA bystander antigen than rCT-B. Although the adjuvant activity of rLT-B in our hands has been highly variable, such results could reflect differences in the receptor binding activities of these proteins (13).

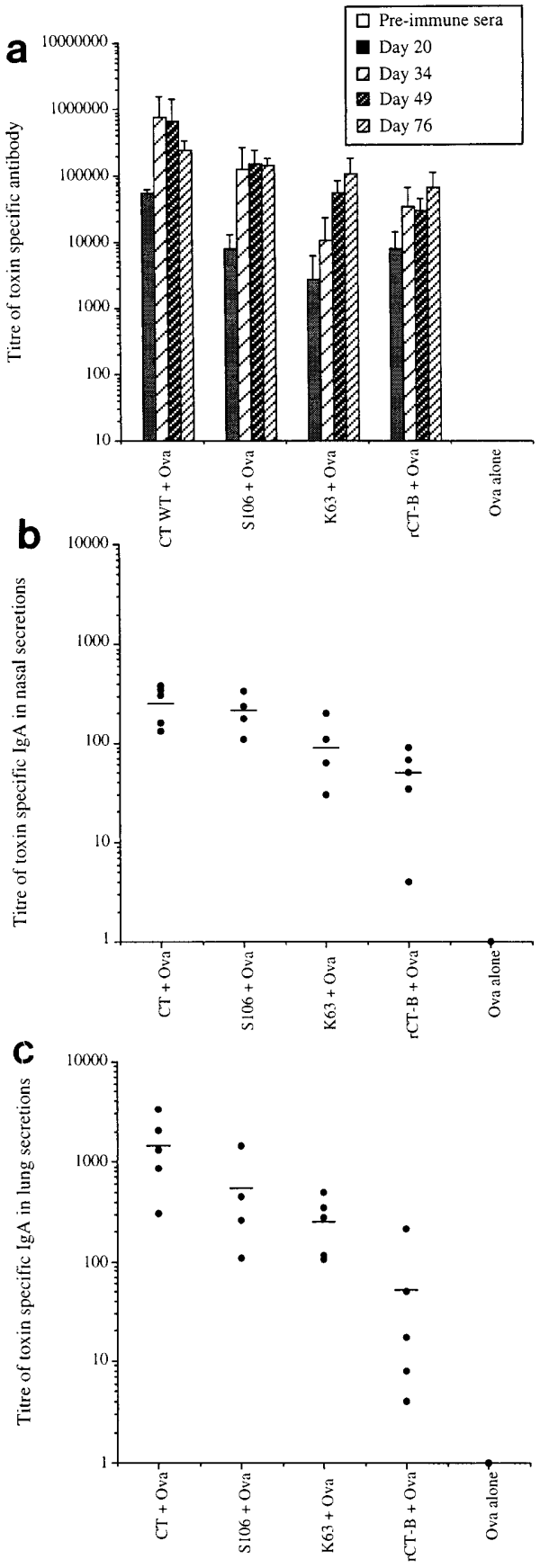
The ability of LTK63 to act as a more efficient adjuvant than CTK63 is also demonstrated by the level of OVA-specific IgA detected in the nasal and lung lavages taken after four i.n. immunizations. OVA-specific IgA was not detected in any of the mice immunized with CTK63 as the adjuvant (Fig. 5a); in contrast, anti-OVA IgA antibody was detected in both the lung and nasal washes from nearly all mice immunized with LTK63 as the adjuvant (Fig. 5b). However, the magnitude of the anti-OVA IgA response was much more variable than the responses observed in mice immunized with wild-type LT. The consistent data generated from samples taken from mice immunized with wild-type toxins may reflect the ability of these mutants not only to bind but also to then enhance immune responses by the presence of the ADP-ribosylation activity.

Antibody subclass responses to coadministered bystander antigen and to toxin derivatives following mucosal immunization. Sera prepared from the final blood samples from immunized mice were used to determine IgG subclass responses to bystander antigens following i.n. immunization. IgG subclass responses induced to OVA bystander antigen with CT and LT as i.n. adjuvants and to the toxin are shown in Fig. 6a and b, respectively. Coadministration of OVA with wild-type CT induced a predominance of OVA-specific IgG1 and IgG2b antibodies. This pattern of response was found in every individual mouse tested. In contrast, coadministration of OVA with wild-type LT induced a higher level of IgG2a and a lower level of IgG1 and IgG2b. However, the pattern of response was very variable between individual animals, suggesting a more mixed response. The data for CT are very similar to other previously reported results (27). Subclass responses to both OVA and the toxin in the sera of mice immunized with the different toxin derivatives were also measured. In each case, the pattern of response was equivalent to that observed when the native toxin was used, although in each case, the titer was lower.

DISCUSSION

We have explored a number of different site-directed mutants of CT and LT to examine the relationship between toxicity and i.n. adjuvanticity. Here, we have detected an apparent correlation between the ADP-ribosyltransferase activity of CT and the potency of the protein as an i.n. adjuvant. The non-ADP-ribosylating derivatives CTK63 and rCT-B were poor i.n. adjuvants compared to wild-type CT. CTK63 and CT-B induced only low levels of bystander-specific antibody in the serum of mice and no significant specific IgA response in either the nasal or pulmonary secretions. In contrast, mutant CTS106, which retained a low level of ADP-ribosyltransferase activity, induced significantly higher levels of both serum and mucosal antibody to bystander proteins than CTK63, suggesting that ADP-ribosyltransferase activity enhances the immune responses required for early adjuvanticity.

Previously, we have demonstrated that nontoxic mutants of LT, such as LTK7 (9) and LTK63 (7), are able to act as adjuvants to bystander proteins when presented i.n. As with CT, the mutant LT derivatives were not as effective mucosal adjuvants as the native LT was (9). Nevertheless, in a direct comparison of the mucosal adjuvant activity of LTK63 and CTK63, LTK63 was a better mucosal adjuvant than CTK63, at least for the bystander antigens tested and the mode of immunization used here. This raises the question of how two highly homologous proteins induce such different responses. One explanation is that there may be significant differences in the



stability or persistence of the two proteins in vivo. From our work with a variety of LT mutants, we are aware that the introduction of some mutations within the A subunit generate proteins which are more susceptible to proteases such as trypsin, possibly because the mutation reduces the stable interaction of the A subunit with the B subunit (21). This work showed that there was a direct correlation between the efficiency of holotoxin formation and the stability of the holotoxin structure. While a mutation in position 63 in LT resulted in 98% holotoxin formation (native LT toxin, 93%), the same mutation in CT produced a derivative which assembled holotoxin less efficiently (82% for CTK63 and 83% for the native CT). This variation in stability may also occur in vivo at sites where immunity is induced; however, this is difficult to measure accurately. The evidence that CTK63 is less immunogenic than wild-type CT lends support to this theory since CTK63 may be more unstable in vivo than either CT or LTK63. Alternatively, the serine at position 63 in CT may make a more significant, but to date unknown, contribution to mucosal adjuvanticity than the equivalent amino acid in LT. We have also identified other site-directed mutants of LT that can still form LT holotoxoid but which have mucosal adjuvanticity equivalent to LT-B (unpublished data). Thus, the combination of the individual mutation and the toxin backbone (LT or CT) may greatly influence stability, immunogenicity, or adjuvanticity. For example, we cannot rule out the possibility that some nontoxic mutants of CT may retain mucosal adjuvanticity. Lycke et al. (19), using oral immunization, showed that a non-ADP-ribosylating LT derivative harboring a mutation in position 112 of the A subunit was still able to form holotoxin but lacked detectable adjuvant activity in mice. However, the selection of the route of immunization may also be critical for demonstrating mucosal adjuvant activity. We have previously shown that the i.n. route of immunization is much more sensitive, at least in mice, than the oral route, where much higher levels of adjuvant and bystander are required to induce antibody production locally or in the serum (8). We do not know whether LT112 can act as a mucosal adjuvant if delivered i.n.

CT and LT may activate immune responses to bystander proteins via different mechanisms. This theory is supported by the fact that wild-type LT and CT induce measurably different immune responses in vivo. Wild-type LT and CT induced different IgG subclass antibody profiles in the serum to OVA. The predominance of IgG1 and IgG2b antibodies to OVA in mice immunized i.n. with wild-type CT as the adjuvant suggests that a Th2-type T-cell help is induced, while the dominance of IgG2a in mice immunized with LT suggests a skew toward a mixed or more Th1-type of response. These general patterns of T-cell help induced by CT and LT, respectively, have also been described by other workers who have studied the cytokines released by T cells taken from mice following immunization with either wild-type CT or LT as a mucosal adjuvant (23, 35). Study of the literature suggests that many different immunological phenomena in CT activate adjuvanticity, including production of active interleukin-1 (IL-1) (1), cyclic AMP-driven switching from IgM-producing B cells to IgA and IgG production (20), down-regulation of IL-2 production from T cells, and

FIG. 3. i.n. immunogenicity of genetic derivatives of CT. (a) Toxin-specific response in the sera of mice. The mean titers of toxin-specific antibody in the sera of five mice taken on days 0, 20, 34, 49, and 76 are shown. Error bars indicate the standard deviations from the mean titer for five mice. (b) Titers of toxin-specific IgA in nasal lavages from the same mice as those described for panel a on day 76. (c) Titers of toxin-specific IgA in the pulmonary lavages from the same mice as those described for panel a.

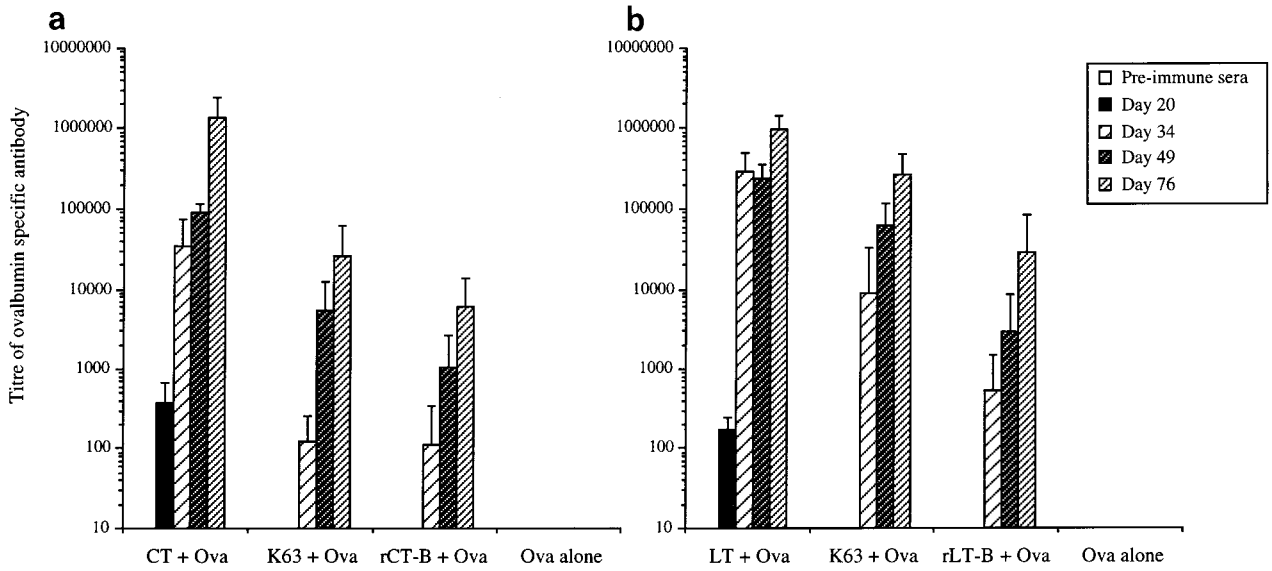


FIG. 4. OVA-specific response in the sera of mice immunized i.n. with either native LT, CT, or detoxified derivatives of both toxins. (a) Mean titers of OVA-specific antibody in the sera of five mice immunized with CT or its detoxified derivatives taken on days 0, 20, 34, 49, and 76; (b) mean titers of OVA-specific antibody in the sera of five mice immunized with LT or its detoxified derivatives which were immunized and bled at the same time. Error bars indicate the standard deviations of the mean titer from five mice.

a strong requirement for IL-4 (39) and CD4 immune cells (14). Significantly less work has been performed with LT, although recent studies have started to monitor the cytokines released from T cells from mice following oral immunization with LT. LT-stimulated T cells release a mixture of IL-2, gamma interferon, and IL-5 and low but detectable levels of IL-4, again indicating a mixed immune response (24). The possible explanation for these differences is that the nature of the immune response induced following mucosal vaccination is influenced by the binding of the toxins to different receptors. CT binds exclusively to ganglioside GM1, while LT is able to bind to GM1 and several other carbohydrate ligands (33). However, experimental analysis of appropriate B-subunit mutants is required to evaluate this possibility.

Our current understanding of the molecular mechanism by which these toxins induce immune responses in vivo is very limited. More in situ studies monitoring the changes in cell activation states, cell populations, and physical organization within local immune inductive sites during an ongoing immune response are required. The abilities of the wild-type toxins and the nontoxic derivatives to act as i.n. adjuvants clearly differ. ADP-ribosyltransferase activity is obviously important for adjuvanticity, although our data would suggest that this activity

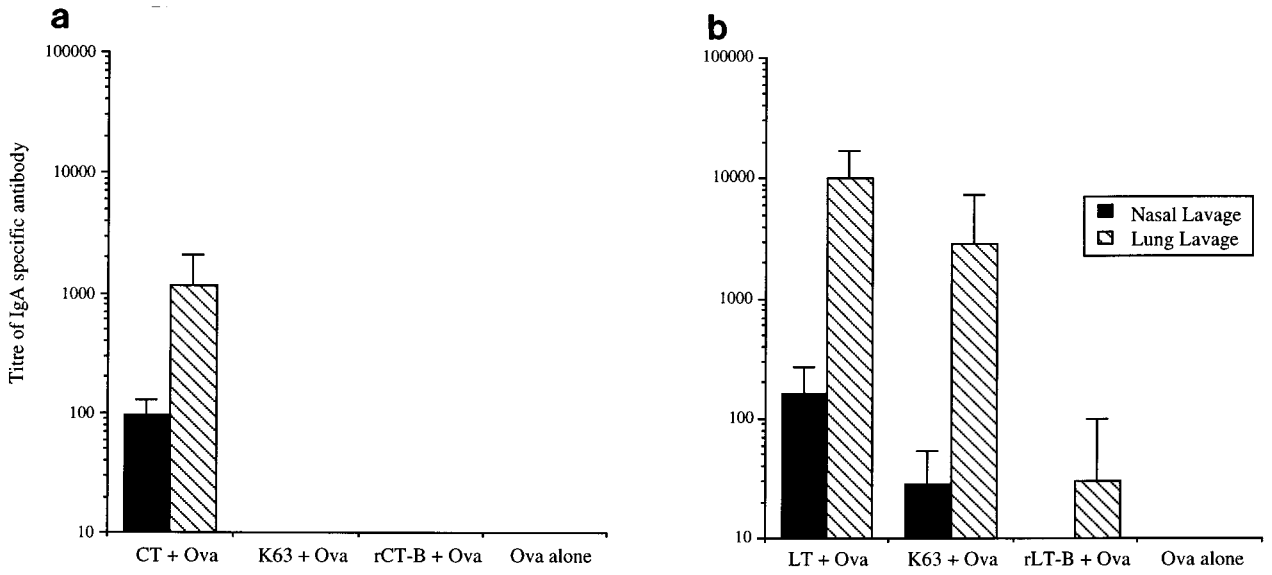


FIG. 5. OVA-specific response in the nasal and pulmonary lavage from mice immunized i.n. with either native LT, CT, or detoxified derivatives of both toxins. (a) Mean titers of OVA-specific IgA antibody in the nasal and pulmonary lavages; (b) mean titers of OVA-specific IgA antibody in the lavages of mice immunized with LT or its nontoxic derivatives. Error bars indicate the standard deviations of the mean titer from five mice.

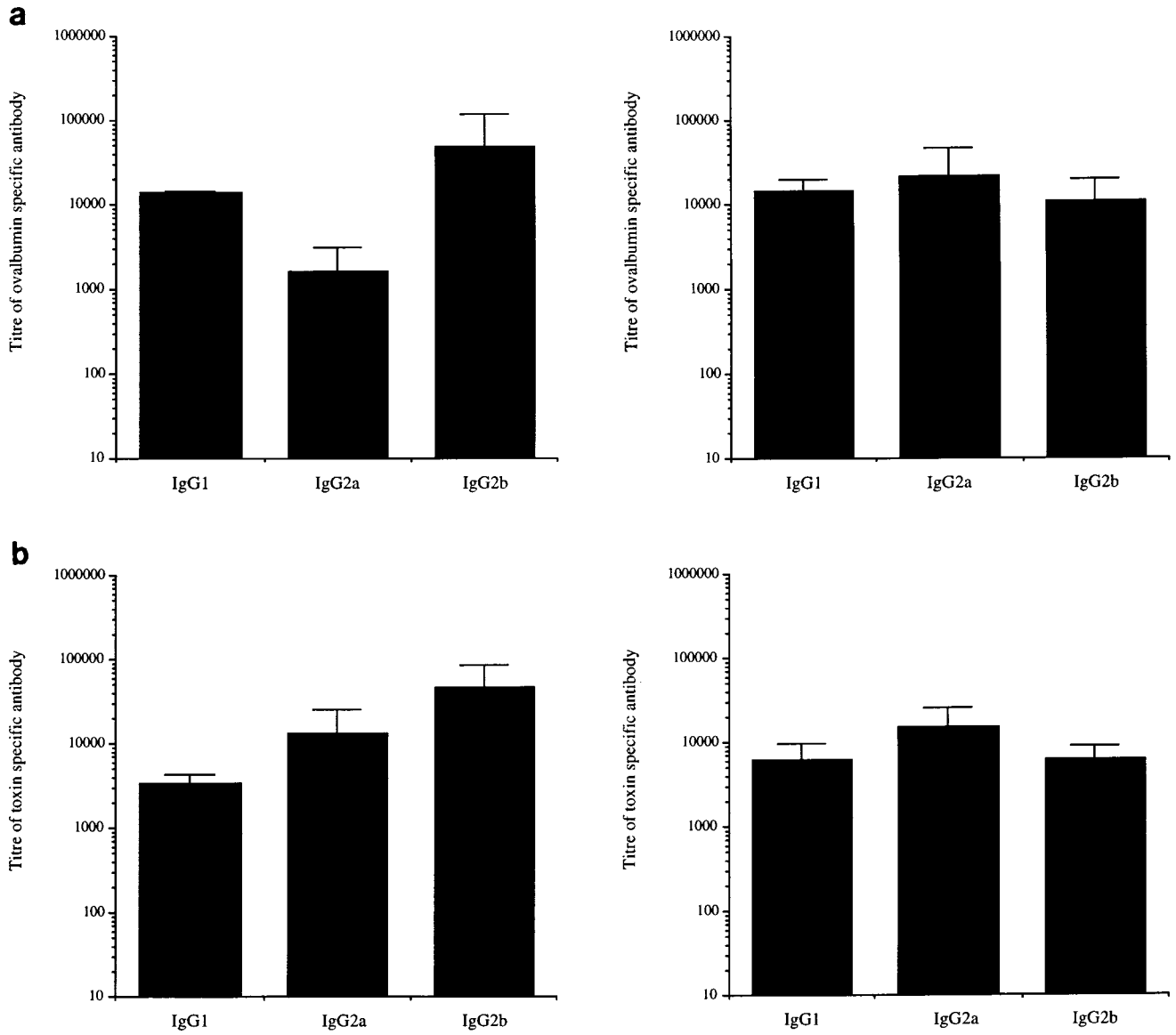


FIG. 6. Subclasses of IgG present in terminal blood samples of mice immunized i.n. with either native CT and OVA or native LT and OVA. (a) Pattern of anti-OVA IgG subclass responses induced in the presence of either native CT (left panel) or LT (right panel); (b) pattern of IgG subclass responses induced to toxin (either native CT [left panel] or LT [right panel]). Titers were calculated by using dilutions of pooled sera, studied in duplicate.

maybe more important for the adjuvant activity of CT than LT. What are the steps required to induce mucosal adjuvant activity? Data from using different routes of immunization suggest that the amount of bystander actually reaching the immune inductive site may be important. For example, the oral route requires more protein than the i.n. route does (8). In addition, the inherent immunogenicity of the bystander protein may be important. In our hands, bystander immune responses to fragment C are induced fairly easily, responses to keyhole limpet hemocyanin require an extra dose, whereas immune responses to OVA are the most difficult to achieve, requiring several i.n. doses. The ability of the toxins to bind to target cells is also required for their inherent mucosal immunogenicity, at least for LT and other ADP-ribosylating toxins such as pertussis toxin (3). The presence of the A subunit associated with the B subunit may increase in vivo stability, but the presence of even an inactive A subunit may influence intracellular events, following toxin interaction with eucaryotic cells, that enhance immunogenicity or adjuvant activity by unknown mechanisms. The presence of an active A subunit dramatically enhances adjuvant activity, particularly following the administration of single doses of vaccine (9). Thus, the presence of ADP-ribosyltransferase activity may serve to amplify local immune inductive events mediated by the binding of the B subunit, the A-B complex, or along a particular pathway.

Together, these data suggest that although CT and LT are highly homologous, they should not be considered as identical proteins in immunological terms. However, by the use of mutants of both CT and LT which retain different biological properties, it may be possible to dissect out the immunological steps which are important in the induction of mucosal immune responses to bystander antigens. Such information will be an

essential requirement in the drive toward identifying more effective mucosal adjuvants.

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