Detoxification of Cholera Toxin without Removal of Its Immunoadjuvanticity by the Addition of (STa-related) Peptides to the Catalytic Subunit

A POTENTIAL NEW STRATEGY TO GENERATE IMMUNOSTIMULANTS FOR VACCINATION*

Peptides related to the heat-stable enterotoxin STa were fused to the N terminus of the A-subunit of cholera toxin (CTA) to explore whether peptide additions could help generate detoxified cholera toxin (CT) derivatives. Proteins carrying APRPGP (6-CTA), ARSCELCNPACPP (16-CTA), or AASSNYCCELCCNPACTGCYPGP (23-CTA) were genetically constructed. Using a two-plasmid system these derivatives were co-expressed in *Vibrio cholerae* with cholera toxin B-subunit (CTB) to allow formation and secretion of holotoxin-like molecules (engineered CT, eCTs). Purified eCTs maintained all normal CT properties yet they were more than 10-fold (eCT-6), 100-fold (eCT-16), or 1000-fold (eCT-23) less enterotoxic than wild-type CT. The inverse correlation between enterotoxicity and peptide length indicated sterical interference with the ADP-ribosylating active site in CTA. This interpretation agreed with greater than 1000-fold reductions in cAMP induction, with reductions, albeit not proportional, in *in vitro* agmatine ADP-ribosylation, and was supported by molecular simulations. Intranasal immunization of mice demonstrated that eCTs retained their inherent immunogenicity and ability to potentiate immune responses to a co-administered heterologous protein antigen, although in variable degrees. Therefore, the addition of STa-related peptides to CTA reduced the toxicity of CT while partly preserving its natural immunoadjuvanticity. These results suggest peptide extensions to CTA are a useful alternative to site-directed mutagenesis to detoxify CT. The simplicity of the procedure, combined with efficient expression and assembly of derivatives, suggests this approach could allow for large scale production of detoxified, yet immunologically active CT molecules.

Cholera toxin (CT) is the archetype bacterial enterotoxin with an A-SB subunit protein structure (1). The B-subunit (CTB), of 103 amino acids, forms a homopentamer that associates with the A-subunit (CTA), of 240 amino acids, to form a holotoxin. The crystal structure of CT (2) shows the CTA pentamer to be a symmetric protein structure with a doughnut-like shape. The central pore in this doughnut-like structure holds CTB by noncovalent interactions.

Cell intoxication by CT requires binding of the holotoxin to GM₁ ganglioside receptors on the cell surface (3, 4). Pentameric CTB is responsible for interactions with GM₁ and five binding pockets have been identified, one per each CTB monomer (5). After binding to GM₁, the holotoxin is endocytosed (6) and CTA becomes dissociated from the CTB pentamer in a late endosomal or trans-Golgi compartment and is then channeled to the endoplasmic reticulum by retrograde transport (7, 8). Following these events the CTA is translocated to the cytoplasm by an as yet poorly defined mechanism. Once in the cytoplasm, CTA enzymatically cleaves NAD⁺ and attaches the ADP-ribose moiety to the G₉₀ component of adenylyl cyclase, causing an increase in the intracellular levels of cAMP (9). This process is stimulated by endogenous proteins designated ADP-ribosylation factors (10). The ADP-ribosylation activity of CTA is increased when the polypeptide chain is proteolytically cleaved, at or around position 192, to give two polypeptides designated CTA1 and CTA2 (11). CTA1 is the enzymatically active portion of CTA and does not significantly interact with the CTB pentamer because interactions with the highly charged central pore in CTB are established by the C-terminal CTA2 polypeptide.

Crystallographic studies have revealed that, in the assembled holotoxin, the ADP-ribosylation active site in CTA1 is located at the top of the molecule (2, 5), if taking the binding sites for GM₁ as the bottom part of the holotoxin. Catalytically active residues in CTA1 have been located primarily through structural comparisons with other ADP-ribosylating toxins.

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such as exotoxin A from *Pseudomonas aeruginosa* (12) and with the structurally and functionally highly related heat-labile enterotoxin (LT) of *Escherichia coli* (13). Based on such comparisons, and on mutagenesis studies, a number of residues can be identified in CT as necessary either from a structural and/or catalytic point of view, including Arg-7, His-44, Ser-61, Ser-63, Ala-72, Glu-110, and Glu-112 (14–22). In addition, the conformation of a loop, constituted by residues 47–56, appears important for the catalytic activity of the A1 domain because in LT a substantial rearrangement of it must occur to accommodate NAD$^+$ (23).

Site-directed mutagenesis targeting one or more of the above CTA residues has had as its primary goal the knocking-out of the ADP-ribosylation activity to detoxify CT. Detoxified CT (or LT) is of great interest in vaccine development because it could potentially be both a good mucosal immunogen for induction of specific antitoxic immunity against cholera or ETEC diarrhea, and as an adjuvant that could potentiate immune responses against co-administered heterologous antigens (24).

In our preliminary work (25), we have found that the addition of an analogue of the heat-stable enterotoxin STa of *E. coli* to CTA caused reductions in CT enterotoxicity. Based on those results, we decided to explore this as an alternative to site-directed mutagenesis for detoxification of CT. Accordingly, we placed various STa-related peptide extensions at the CTA N terminus to then evaluate their effect over the toxic, immunogenic, and immunoadjuvant properties of CT.

We here show that the generated CTA fusion proteins efficiently assemble with CTB to form modified engineered CT-like molecules (referred to as eCTs). Further, we demonstrate that the produced molecules are detoxified partly or fully and, because detoxification correlated with peptide length, we propose that it was primarily caused by sterical interference of the added peptides with the ADP-ribosylating active site. Despite detoxification the eCTs were strongly immunogenic as well as immunoadjuvant-active in mice upon intranasal-mucosal immunization. These results indicate that addition of peptides to CTA may be a useful approach to produce detoxified, yet immunogenic and immunoadjuvant-active CT derivatives.

**EXPERIMENTAL PROCEDURES**

**Genetic Constructions**—The CTA gene as encoded by plasmid pRA003-STD (25) was inserted into the pUC18 polylinker (26) and then fused to paired oligonucleotides encoding ASRCAELCCNPACAP. These cloning steps generated plasmid pJS814, which directed the synthesis of the fusion protein ASRCAELCCNPACAP-CTA, designated 16-CTA. In plasmid pJS814, as well as in the constructs derived from it, the natural CTA leader peptide was replaced by the leader peptide of the LT B-subunit (27) and the different recombinant CTA genes were expressed from the lac promoter (26). Use of the sequence encoding the LT B-subunit leader peptide, including the ribosome-binding site, has earlier provided with high expression levels of CTB in *E. coli* (28). This seems to also have been the case for CTA, although this was not formally proven. The insert in pJS814 encoding the 16-amino acid peptide was then replaced using PCR by a sequence now encoding APRPGP. The resulting plasmid, designated pJS815, encoded for the fusion protein APRPGP-CTA (6-CTA). In plasmid pJS815 there were single SacI and XmaI sites flanking the new DNA insert, and these restriction sites were used to replace APRPGP by synthetic paired oligonucleotides encoding ANSSNYCCELCCNPACTGCYPGP, the sequence for mature human STa of *E. coli* heat-stable enterotoxin (29, 30). The plasmid encoding ANSSNYCCELCCNPACTGCYPGP-CTA was designated pJS816, and the respective fusion protein was named 23-CTA.

**Expression, Purification, and Biochemical Characterization of Holotoxins**—Plasmids pJS815 (6-CTA), pJS814 (16-CTA), and pJS816 (23-CTA) were independently electrotransported into the CTA-depleted *Vibrio cholerae* host strain JS1569 (28). Thereafter, a companion compatible plasmid pJS384 encoding recombinant CTB (25) was introduced for genetic in trans complementation. The use of this two-plasmid system led to efficient assembly and secretion of eCTs into the culture supernatant with an average yield of 25 μg of eCT/ml of culture medium. For expression of eCTs, 2.5-liter Luria Broth liquid cultures supplemented with ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), and isopropyl-β-D-galactopyranoside (100 μg/ml) were grown under shaking at 37°C for 18 h. The expressed eCTs were then isolated from culture supernatants by sodium hexametaphosphate precipitation (31) followed by dialysis of precipitates against phosphate-buffered saline and twice FPLC fractionation on Superdex 5-75 columns (Amersham Biosciences AB, Uppsala, Sweden). This procedure resulted in isolation of ~10 mg of highly purified eCTs/dilute of starting culture media.

For characterization, the purified eCTs were first tested by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and protein bands stained with Coomassie Blue. Commercial wild-type CT (List Biological Laboratories) was used as a standard for protein size comparisons. Western blot assays using anti-CTA (32) or anti-STa (33) monoclonal antibodies were also done on SDS-PAGE separated proteins. The epitope recognized by anti-CTA has not been precisely mapped, but it is confined to the CTA1 peptide, and this allowed for detection of constructs under reducing and denaturing conditions that led to the absence of the CTA2 peptide.

The GM1-ELISA method for detection of CT (34) was here used to test for STa-like molecules with CTB as well as to demonstrate peptide carriage by CTA. Accordingly, purified eCTs were first bound to GM1-coated plates and then separately reacted with anti-CTB (32), anti-CTA, or anti-STa monoclonal antibodies (33) followed by standard ELISA development reactions with goat anti-mouse immunoglobulin-enzyme conjugate and enzyme substrate.

To assay for residual ADP-ribosylation activity, purified eCTs were tested for agmatine-dependent ADP-ribosylation essentially as described by others (35). To estimate reductions in toxicity, constructs were tested at various concentrations in the presence of 14C-labeled NAD (10 μM), 10 mM arginine, 10 mM dithiothreitol, 5 mM MgCl$_2$, 200 μM GTP, and 0.1 mg/ml ovalbumin. Reactions were carried out in a 10-μl volume for 2 h at 30°C. At the end of the incubation period, samples were loaded onto an ion exchange column to retain unincorporated label and radioactivity in effluents (1 ml) measured by liquid scintillation.

Toxicity Assays—The biologic-toxic activity of eCTs was estimated by their capacity to induce cAMP in tissue culture cells and by their enterotoxic-diarrheogenic activity in a rabbit model.

To test for induction of CAMP accumulation, Chinese hamster ovary (CHO) cells were cultured to confluence in plastic bottles, harvested by classical methods, and then seeded onto 96-well plates just prior to the assay. Purified eCTs were then added in concentrations from 10 to 0.01 μg/ml to wells and cells incubated for periods of 3.5, 6.5, and 24 h. At the end of the exposure period, cells were lysed in situ, cell lysates extracted from the wells, and CAMP measurements carried out using the Biokit kit code RPN225 (Amersham Biosciences) establishing a standard CAMP curve as recommended by the suppliers.

Enterotoxicity tests were carried out in rabbit intestinal loops (RIL) essentially as described (36). For assay, 1-ml samples were tested in duplicate and in concentrations ranging from 10 μg/ml down to 0.001 μg/ml by inoculation into two noncontiguous 5-cm small intestine loops. After 18 h of exposure, fluid accumulation was determined and the average volume-to-loop length ratio calculated. Purified CT was used as positive control and purified recombinant CTB (Ref. 28; provided by SBL Vaccine, Stockholm, Sweden) and phosphate-buffered saline as negative controls. Tests for STa toxic activity were done in infant mice with the eCT23 at a concentration of 10 μg/ml in 100-μl volumes.

**Immunoassays**—Groups of five BALB/c mice were immunized intranasally with the various eCTs together with ovalbumin (OVA). Twenty μg of OVA were given in 10-μl volumes to lightly anesthetized animals either alone or together with 1 or 10 μg of each eCT, with 1 μg of reference CT or 1 or 100 μg of recombinant CTB. At 2 and 4 weeks, identical booster immunizations were given; after 2 more weeks, the mice were sacrificed and serum samples were obtained. Samples were assayed by ELISA for anti-OVA or anti-CTB activity using either the eCT or CT as coating antigens. Positive control sera for each of these antigens were concomitantly tested. Mean values and standard deviations for each group were calculated and results expressed in logarithmic form.

**Molecular Modeling**—Theoretical molecular models for the fusion proteins with the various peptide extensions at the N terminus of CTA were constructed with the Quantum97/CHARMM22 modeling package.
Potential Novel Strategy to Detoxify Cholera Toxin

C.T. constructs were carried out mainly to study local conformational surrounding the N terminus of CTA were found. Only minor perturbations of the side chains and after addition to the N terminus of CTA (Protein Data Bank entry 1XTC) (2), having conformations allowing maximal reach up toward the putative catalytic cleft. Furthermore, retained antibody binding (40) supports the supposition that the disulfide bridges actually are formed by cysteines 6:11, 7:15, to the appearance of the three-dimensional structure, which reveals disulfide bridges 6:11 and 10:15. This modification was performed prior changed to Ala (39). This was calculated to allow for formation of the two remaining disulfide bridges, Cys-7 was changed to Ala (39). This was calculated to allow formation of disulfide bridges 6:11 and 10:15. This modification was performed prior to the appearance of the three-dimensional structure, which reveals that the disulfide bridges actually are formed by cysteines 6:11, 7:15, and 10:18. However, manipulation of the side chain torsion angles of cysteines 10 and 15 shows that these two residues may form a disulfide bridge with very minor perturbations of the remainder of the structure. Furthermore, retained antibody binding (40) supports the supposition that the overall folding is very similar to that of the toxic domain of ST (residues 5–17).

Finally, the smallest peptide extension (6-CTA) was simply built as an extended chain for analysis. All three extensions were subject to energy refinement both prior to and after addition to the N terminus of CTA (Protein Data Bank entry 1XTC) (2), having conformations allowing maximal reach up toward the putative catalytic cleft. Only minor perturbations of the side chains surrounding the N terminus of CTA were found.

Molecular dynamics simulations (200 ps) in vacuum for the three CTA constructs were carried out mainly to study local conformational properties of the proteins to allow the different extensions to assume probable interactions with the CTA molecular surface.

Apart from the extensions themselves, all residues on CTA corresponding to regions for which interactions might occur were allowed freedom of movement. A distance-dependent dielectric constant (\(\varepsilon = 6\)) was used for the simulations, and the SHARE algorithm was used to constrain bonds containing hydrogen atoms, allowing a time step of 2 fs. Simulations were started by heating the system from 0 K up to the final temperature (300 K) using a 5-K increment every 100 time steps, followed by an equilibration period of 10 ps.

RESULTS

Assembly of Peptide-extended CTA Fusion Proteins with CTB—Convincing evidence for assembly of peptide-extended CTA-fusion proteins with CTB was provided by comparison of FPLC profiles of the different eCTs with those for unmodified CT, pentameric CTB, and isolated CTA (Fig. 1). The eCT6 and eCT23 gave single peaks, whereas there was a "shoulder" for the eCT16. The appearance of this shoulder was attributable to a modest degree of spontaneous dissociation into pentameric CTB and free (peptide-extended) CTA. Lower stability of this eCT was confirmed by showing that repeated freezing and thawing caused an increase in the height of the peak and a lower Gm1-ELISA titer when assaying for assembled (see below) eCT.

Further evidence for assembly of the different CTA fusion proteins into holotoxin-like molecules and for the carriage of peptide extensions by CTA was provided by SDS-PAGE, Western blots analyses, and Gm1-ELISA tests.

After separation of CTA during SDS-PAGE (Fig. 2A) and reaction with specific antibodies in Western blot assays, 6-CTA, 16-CTA, and 23-CTA subunit proteins reacted with an anti-CTA antibody (Fig. 2B) and the position of immunoreactive bands showed a slightly slower migration than that for unmodified CTA. The 16-CTA and 23-CTA proteins also reacted with anti-STa monoclonal antibodies in support of
their carriage of the expected STa-related peptides (Fig. 2B). The G\textsubscript{M1}-ELISA test served to support both the assembly of peptide-extended CTA proteins with CTB and carriage of appropriate peptides. In the G\textsubscript{M1}-ELISA, the CTA subunit is detected only when associated to CTB because binding to G\textsubscript{M1} depends upon the CTB pentamer (1). Therefore, the positive reaction with anti-CTA in G\textsubscript{M1}-ELISA (Fig. 3A) constitutes proof that CTA derivatives in the eCTs are assembled with the CTB pentamer in a fashion that did not interfere with the latter moiety binding to G\textsubscript{M1} (and then most likely also to cells). Interestingly, even though concentrations of eCTs were adjusted to the same value, some differential reactivity of the three constructs was observed (Fig. 3A). Differences in reactivity in the G\textsubscript{M1}-ELISA could be caused by differently efficient binding to G\textsubscript{M1} and thus reflect variable amounts of bound eCT; however, reactions with anti-CTB monoclonal antibody showed essentially identical reactivity in the various samples (data not shown). We therefore attribute the differential reactivity to unequal accessibility of the anti-CTA epitope rather than differences in G\textsubscript{M1} binding. In the same way that reactivity with anti-CTA could only take place if CTA was associated with CTB, reaction of both the 16-CTA and 23-CTA constructs with anti-STa monoclonal antibody showed essentially identical reactivity in the various samples (data not shown). We therefore attribute the differential reactivity to unequal accessibility of the anti-STa epitope rather than differences in G\textsubscript{M1} binding.

**Induction of cAMP Accumulation in Cells**—To determine the effect of peptide extensions on the toxic-active catalytic activity of CTA in mammalian cells, the capacity of the different eCTs to induce cAMP accumulation in CHO cells was determined in comparison with that of wild-type CT. Various eCT concentrations and incubation times were tested. Results show that all eCTs had very marked reductions in their ability to induce cAMP accumulation being >1,000-fold and up to >100,000-fold less toxic than CT (Fig 4, A–C). Although the cAMP induction capacity of eCTs relative to CT did not essentially change with exposure time, the absolute activity did. Therefore, at the longest 24-h exposure time (Fig. 4C), the activity of the eCT6 and eCT16 was much higher than at 3.5 h and the activity of the eCT23 turned to a measurable value from a totally negative response. A similar delay was not observed for wild-type CT, for which the maximal cAMP induction occurred between 3.5 and 6.5 h (Fig 4).

**Toxicity of eCTs**—To find out whether reductions in cAMP accumulation were reflected in lower enterotoxicity of eCTs, RIL assays were conducted. The RIL tests showed the eCT6 to be substantially (10-fold) less toxic than CT, the eCT16 to be ~100-fold less toxic, and the eCT23 to display no activity even at the highest dose tested (100 \( \mu \)g/ml), thus being >1000-fold less active than wild-type CT (Table I). The RIL results correlated with cAMP induction tests and suggested that the addition of peptides to the N terminus of CTA reduced the enterotoxic activity of CT because of diminished CTA catalytic activity. As observed for cAMP induction, decreases in enterotoxicity were found to also correlate inversely with the length of the added peptide. The eCT23 were also tested for STa enterotoxic activity at 10 \( \mu \)g/ml (0.1 ml/dose) in infant mice and gave a negative result.

**In Vitro ADP-ribosylation**—To determine the residual ADP-ribosylation activity in eCT constructs, ADP-ribosylation of agmatine was assayed. The ADP-ribosylating activity of eCTs was compared with that of wild-type CT at different toxin concentrations (Fig. 5). Results show reductions in ADP-ribosylating capacity that also correlated with the length of the added peptide. However, decreases in ADP-ribosylation were much smaller than those observed in cAMP or in enterotoxic activity. Agmatine ADP-ribosylation was dose-dependent, but at lower concentrations differences between eCTs and CT tended to be increased. For example, at the highest (5 \( \mu \)g) amount of toxin, the eCT23 was approximately 12-fold lower in activity than wild-type CT, whereas at the lowest amount (0.2 \( \mu \)g) this difference turned to ~30-fold. These results suggested that activity may be dependent upon the toxin-to-substrate ratio.

To test whether, after administration to mice, eCTs could be processed so that added peptides were removed by host proteases, the eCT23 protein was treated with trypsin (2 \( \mu \)g/10 \( \mu \)g of eCT), but we detected no difference in ADP-ribosylation.

**Fig. 3.** G\textsubscript{M1}-ELISA showing assembly into eCTs and evidence of peptide carriage by CTA. Each purified eCT was 3-fold serially diluted in situ in 96-well G\textsubscript{M1}-coated plates and then reacted either with anti-CTA (A) or with anti-STa (B) monoclonal antibodies. After development with peroxidase substrate (OPD), the absorbance at 450 nm was measured and then plotted (y axis) against the respective dilution (x axis). A, demonstration of assembly of the various CTA fusion proteins with CTB by reaction with anti-CTA. B, reaction with anti-STa to demonstrate both assembly with CTB and carriage of STa-related peptides. As expected, the eCT6 did not give a positive reaction with anti-STa (data not shown).
activity (data not shown). Similarly, when eCT16 was inoculated into rabbit ileal loops and accumulated fluid recovered to assay for residual eCT and released immunoactive STa peptide by GM1-ELISA, we did not detect processing of the added peptide (data not shown). This indicates that the junction between the added peptides and the N terminus of CTA is not especially prone to proteolytic cleavage.

Mucosal Immunogenicity and Adjuvanticity of eCTs—To determine whether the peptide extensions to CTA with their marked effect on toxicity had affected the inherent mucosal immunogenicity of the corresponding eCTs, we studied the anti-CTB and anti-CTA antibody serum responses elicited after immunization of mice via the nasal route. Results show that all three eCTs retained good immunogenicity, as estimated by the anti-CTB IgG levels in serum (Fig. 6A). At the same time, the results clearly showed, as tested with a low dose (1 μg) immunization regimen, that there was an inverse relation between the immunogenicity and the reduction in enzymatic-toxic activity caused by the different peptide extensions to CTA, the eCT6 being best immunogenic and the eCT23 being the least active. Still, the latter molecule was significantly more immunogenic than CTB itself. Increasing the dosage of the eCTs resulted in much higher immune responses, such that now even the almost completely nontoxic eCT23 had similar immunogenicity as 1 μg of CT (Fig. 6A).

The anti-CTA antibody response patterns closely resembled the anti-CTB responses albeit at ~2 orders of magnitude lower levels (data not shown).

To determine whether detoxification of CT, as evidenced by reductions in enterotoxicity and in cAMP induction, still allowed for expression of the known ability of CT to serve as an adjuvant for co-administered protein antigens, mice were intranasally immunized with OVA antigen in combination with the various eCTs at 1- and 10-μg doses. Fig. 6B shows that anti-OVA IgG serum antibody levels were raised by all three eCTs to a greater extent than obtained with CTB, and that the eCT6 gave rise to titers close to those obtained with wild-type CT as adjuvant. A dosage-dependent response was observed also for the adjuvant activity, as all three eCTs induced higher anti-OVA titers when given in 10-μg as compared with 1-μg doses (Fig. 6B). Comparison of the adjuvanticity with recombinant CTB at a 100-μg dose demonstrated that results were not simply the consequence of the weak adjuvant action of the CTB component in eCTs.

Structural Simulations—The dramatically reduced induction of cAMP in tissue culture cells and the observed decrease in the in vivo enterotoxicity and in agmatine ADP-ribosylation were consistent with interference of the fused peptide extensions with the ADP-ribosylation catalytic site in CTA. To explore this possibility in more depth, dynamics simulations of the A1 domain of CTA carrying the different peptide extensions were done.

In all three CTA constructs, the sequences Pro-Ala-Pro or Pro-Gly-Pro, which act as connectors between the extension peptide and CTA, are present. The restrictions imposed by these Pro-X-Pro sequences, as well as that by the upper face of pentameric CTB, gave the peptide extensions orientations in which their long axes were roughly perpendicular to the upper CTB surface but initially somewhat removed from the A1 surface. However, close contacts between the different peptide extensions and the A1 domain were quickly established, as were also the final conformations. Although some variations could be observed depending on the starting conditions, the small differences found do not alter the general conclusion from the simulations that only relatively minor structural perturbations of the A1 domain were caused by the peptide extensions even when allowing the whole structure to be movable.

In the structural model for 23-CTA, the peptide extension partly occludes the putative active site of the A1 domain and interacts, among others, with residues Ile-64, Ser-65, Asp-109,
Glu-110, and Gln-111, which are situated either in close proximity to the active site or are part thereof. In the 23-CTA only the N- and C-terminal parts of the extension experienced large conformational changes as expected, whereas the folding of the CCELCCNPACTGC domain of the STa sequence remained relatively unchanged by virtue of its three disulfide bridges. As the 23-amino acid extension acquired interactions with the A1 domain, the original N terminus moved upward by 4.5 Å as viewed in Fig. 7.

In the 16-CTA construct, the STa-related domain (decapeptide) of the peptide extension reaches almost as high as the extension in 23-CTA, making contact with Ile-64 and Gln-111 of the A1 domain, but the N-terminal tripeptide is in this case folded to the right and downward (data not shown). However, the STa-related domain in both 16-CTA and 23-CTA interact with A1 with the same amino acid side chains, notably the segment NPAC but in 16-CTA the domain veers to the right by 4–5 Å and rotates clockwise 90° if compared with 23-CTA as shown in Fig. 7 (data not shown).

Finally, for the smallest extension (6-CTA), it was found that the N-terminal residue almost reaches up to Ile-64 but does not appear to directly interfere with any of the active site residues in A1 (data not shown).

DISCUSSION

In this work we have explored the addition of peptides to the CTA N terminus end as an approach to reduce CT enterotoxicity without affecting its natural immunoadjuvant capacity. For the fusion to CTA, we chose sequences that were structurally related and of variable length as follows: ANSSNYCCCELCCNPACTGCYPGP, where underlined amino acids are the STa (or STI) heat-stable enterotoxin from a human E. coli strain (29, 30), ASRCAELCCNPACPAP, where the underlined decapeptide sequence is analogous to the internal toxic core in STa (38) and the sequence APRPGP.

We favored peptide additions over amino acid mutagenesis (14, 15, 20–22) because, in this procedure, the main polypeptide chain would not be altered, and this was theorized to reduce the likelihood of destabilizing the CTA structure. Moreover, because the CTA amino-end in the native toxin is located at ~11 Å from the CTB pentamer (2), we reasoned that the

| Dose (µg) | CT | eCT6 | eCT16 | eCT23 | CTB |
|----------|----|------|-------|-------|-----|
| 10       | ND | ND   | 0.5   | <0.1  | <0.1|
| 1        | 1.7a| 0.7   | <0.1  | <0.1  | <0.1|
| 0.1      | 1.2 | <0.1  | <0.1  | <0.1  | <0.1|
| 0.01     | 0.2 | <0.1  | <0.1  | <0.1  | <0.1|
| 0.001    | <0.1| ND    | ND    | ND    | ND  |

a Fluid accumulation expressed as ml/cm.

Fig. 5. ADP-ribosylation of agmatine by eCTs in comparison with wild-type CT. In the y axis, radioactivity values in effluents (cpm) are shown ± 1 standard deviation (n = 4) for each point. Values reflect labeling of agmatine because of transfer of the [14C]-labeled ADP-riboyl moiety of NAD catalyzed by different amounts of CT or eCTs (x axis).

Fig. 6. Immunogenicity and adjuvanticity of eCTs upon mucosal administration to mice together with an unrelated protein antigen, OVA. In both A and B panels, the y axis (logarithmic scale) shows the serum IgG antibody titers obtained after intranasal immunization of mice with either OVA alone or together with CT, eCTs, or CTB. A, serum anti-CTB (immunogenicity). B, serum anti-OVA (adjuvanticity). Abbreviations in the x axis are: NIL, OVA alone; 6, eCT6; 16, eCT16; 23, eCT23; C7, wild-type cholera toxin; CTB, recombinant CT B-subunit.

Table I. Rabbit intestinal loop assay showing decreased enterotoxicity of eCTs

| Dose (µg) | CT | eCT6 | eCT16 | eCT23 | CTB |
|----------|----|------|-------|-------|-----|
| 10       | ND | ND   | 0.5   | <0.1  | <0.1|
| 1        | 1.7a| 0.7   | <0.1  | <0.1  | <0.1|
| 0.1      | 1.2 | <0.1  | <0.1  | <0.1  | <0.1|
| 0.01     | 0.2 | <0.1  | <0.1  | <0.1  | <0.1|
| 0.001    | <0.1| ND    | ND    | ND    | ND  |

*a Fluid accumulation expressed as ml/cm.
amino-end peptide extensions would be sufficiently far away from the CTB pentamer to not interfere with assembly into holotoxin. Additionally, atomic interactions of the α-amino group of Asn-1 with the rest of the CTA protein are predicted to be very few, if any (2). Therefore, we anticipated that during synthesis folding of the mature CTA polypeptide chain would not be seriously compromised by the presence of the extra peptides.

Our choice of extending CTA with peptide derivatives of STa was based on several considerations and preliminary results, which indicated the feasibility of this approach. First, we have previously shown that STa and its analogues can be genetically linked not only to either the N or C terminus of CTB (39, 40) but also to unrelated proteins such as the E. coli outer membrane protein OmpC (41). This suggested that the highly compact and essentially autonomous folding of the cysteine-rich STa-related peptides had produced very stable structures where neither the synthesis nor folding of the carrier protein was seriously compromised. This was hypothesized to also facilitate their fusion to CTA. Second, we have shown that a monoclonal antibody directed toward STa recognizes STa as well as its genetic fusion analogues (40), and this was deemed highly useful for characterization of potential CTA constructs. Finally, a model for the STa molecular structure has been proposed (37), and we anticipated that available coordinates for that model would allow for in silico modeling of our constructs.

Thus, although from the biochemical point of view, STa-related peptides are far from average, we thought they could nonetheless indicate how the addition of peptides to the N terminus of CTA would affect the toxicity and adjuvanticity of CT. This was particularly so because we expected that fusion peptides should fold independently into stable structures that would leave few, if any, of its constituent amino acids free for chemical rather than steric interactions with CTA.

We here demonstrate the successful linkage of all three peptides to the N terminus of CTA. Upon co-expression with CTB, the generated CTA fusion proteins associated with CTB to form CT-like molecules referred to as eCTs. In the usual fashion for native CT, expression of eCTs in V. cholerae led to their efficient expression and secretion into the culture supernatant, thus simplifying their purification for biochemical and biological characterization. Purified eCTs were shown to retain essentially all key properties of CT—with the exception of toxicity, including the ability to bind to the G_{iα} receptor and to react with anti-CTB, anti-CTA, and anti-STa antibodies. In contrast, the purified eCTs were found to have dramatically reduced toxicity measured both as their ability to induce cAMP accumulation in CHO cells and to induce diarrheal fluid accumulation in rabbit intestinal loops. Noticeably, reductions in cAMP induction and in enterotoxicity were both found to inversely correlate with the length of the added peptides. Based on these observations, we postulate steric interference of the peptide extensions with the ADP-ribosylation active site in CTA1.

The latter hypothesis was supported by molecular simula-
tions, which indicate that all peptide extensions could block the active site of CTA although to variable degrees. A model for the longest 23-amino acid extension is shown as an example in Fig. 7. In the model, the 23-amino acid extension can be seen to partially occlude the active site and even interact with certain active site amino acid residues in CTA1. This double effect could help explain the strong reduction in cAMP induction activity and enterotoxicity displayed by the eCT23 holotoxin.

By similar structural modeling approaches, the 16-amino acid extension was shown to also interfere with the active site but to a lesser extent (data not shown). Fewer interactions would agree with the lesser reduction in enterotoxicity and the somewhat higher cAMP induction capacity of the eCT16 as compared with eCT23.

Finally, the 6-amino acid extension in 6-CTA was shown to have no clearly demonstrable interactions with residues in the active site, although it could probably partially hamper substrate access, and this may explain why the eCT6 experienced only an ~20-fold decrease in enterotoxicity and a proportionally higher capacity to induce CAMP (Fig. 4).

Altogether, the molecular modeling results support the notion that reductions in toxicity could be the result of interference with the catalytic site in CTA1.

Analyses of in vitro agmatine ADP-ribosylation by eCTs also stand up for the notion that peptide extensions indeed decreased the ADP-ribosylating activity and in accordance with peptide length, although the -fold decreases in activity in this assay were substantially smaller than the decreases in either CAMP production or enterotoxicity. Differences between the agmatine and other tests may be caused by one of several factors. In the first place, ADP-ribosylation of the surrogate substrate agmatine may only partly reflect the in vivo activity, as agmatine, the de-carboxylated derivative of arginine, is structurally much simpler than the natural target, the GO protein. Therefore, a soluble small freely moving molecule may more easily fit into the active site even in the presence of the extension peptide. Discordances, where agmatine ADP-ribosylation activity has turned higher than anticipated from intoxication of tissue culture cells, have also been reported by others (42).

Besides effects on the catalytic activity, there is an additional potential consequence of the added peptides over the intracellular trafficking of eCTs. It is plausible that the presence of “foreign” peptides at the CTA N terminus acts to hamper its translocation across the ER membrane and that decreases in CAMP accumulation and/or enterotoxicity are partly caused by reduced transport within the cell. This could simply be the result of physical hindrance because of the larger size of the molecule. Alternatively, it may be that the translocation apparatus preferentially recognizes native CTA.

A practical goal of testing peptide extensions to CTA was to determine whether eCTs with reduced toxicity caused by such extensions would retain whole or part of the potent inherent immunogenicity as well as adjuvant activities of CT beyond the activity resulting simply from the nontoxic CTB moiety. Previous work has shown that both of these closely interrelated immunological properties depend on the enzymatic-toxic activity of CT (43, 44). Although approaches to detoxify CT (or LT) by specific mutagenesis have resulted in some interesting molecules with partly retained adjuvant activity despite marked reductions in toxicity (22), these mutants have been difficult to produce in quantity and often have also had problems with stability. At the same time, it was clear in our case that both immunogenicity and adjuvant activity for a co-administered antigen, OVA, decreased with reductions in toxic activity. Importantly, however, these decreases were not proportional inasmuch as the practically completely detoxified eCT23 had significant adjuvant activity even at the 1-μg dosage when CTB was completely ineffective.

In conclusion, we have shown that the addition of STA-related peptides to the N terminus of CTA can cause detoxification of CT without eliminating either its immunogenicity or its adjuvanticity. Thereby, although in variable degrees, the produced eCTs still induced anti-CTB and anti-CTA antibodies and they also potentiated the immune response toward a heterologous protein antigen when given by the mucosal route.

This novel strategy may have practical implications in the field of vaccine development, as it could provide high yields of detoxified CT molecules with capacity to enhance the immune response against co-administered admixed or linked candidate vaccine antigens and/or pathogenic organisms.

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