Adjuvanticity of the Cholera Toxin A1-Based Gene Fusion Protein, CTA1-DD, Is Critically Dependent on the ADP-Ribosyltransferase and Ig-Binding Activity

Lena C. Ågren, Lena Ekman, Björn Löwenadler, John G. Nedrud and Nils Y. Lycke

*J Immunol* 1999; 162:2432-2440; [http://www.jimmunol.org/content/162/4/2432](http://www.jimmunol.org/content/162/4/2432)

<table>
<thead>
<tr>
<th>References</th>
<th>This article cites 43 articles, 23 of which you can access for free at: <a href="http://www.jimmunol.org/content/162/4/2432.full#ref-list-1">http://www.jimmunol.org/content/162/4/2432.full#ref-list-1</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Subscription</td>
<td>Information about subscribing to <em>The Journal of Immunology</em> is online at: <a href="http://jimmunol.org/subscription">http://jimmunol.org/subscription</a></td>
</tr>
<tr>
<td>Permissions</td>
<td>Submit copyright permission requests at: <a href="http://www.aai.org/About/Publications/JI/copyright.html">http://www.aai.org/About/Publications/JI/copyright.html</a></td>
</tr>
<tr>
<td>Email Alerts</td>
<td>Receive free email-alerts when new articles cite this article. Sign up at: <a href="http://jimmunol.org/alerts">http://jimmunol.org/alerts</a></td>
</tr>
</tbody>
</table>
Adjuvanticity of the Cholera Toxin A1-Based Gene Fusion Protein, CTA1-DD, Is Critically Dependent on the ADP-Ribosyltransferase and Ig-Binding Activity

Lena C. Ågren,* Lena Ekman,* Björn Löwenadler,† John G. Nedrud,‡ and Nils Y. Lycke2*

The ADP-ribosylating enterotoxins, cholera toxin (CT) and Escherichia coli heat-labile toxin, are among the most powerful immunogens and adjuvants yet described. An innate problem, however, is their strong toxic effects, largely due to their promiscuous binding to all nucleated cells via their B subunits. Notwithstanding this, their exceptional immunomodulating ability is attracting increasing attention for use in systemic and mucosal vaccines. Whereas others have separated adjuvanticity from toxicity by disrupting the enzymatic activity of the A1 subunit by site-directed mutagenesis, we have constructed a nontoxic molecule that combines the full enzymatic activity of the A1 subunit with a B cell targeting moiety in a gene fusion protein, the CTA1-DD adjuvant. Despite its more selective binding properties, we found comparable adjuvant effects of the novel CTA1-DD adjuvant to that of CT. Here we unequivocally demonstrate, using a panel of mutant CTA1-DD molecules, that the immunomodulating ability of CTA1-DD is dependent on both an intact enzymatic activity and the Ig-binding ability of the DD dimer. Both agents, CT and CTA1-DD, ADP-ribosylate intact B cells. However, contrary to CT, no increase in intracellular cyclic AMP in the targeted cells was detected, suggesting that cyclic AMP may not be important for adjuvanticity. Most remarkably, CTA1-DD achieves similar immunomodulating effects to CT using a ganglioside-GM1 receptor-independent pathway for internalization. The Journal of Immunology, 1999, 162: 2432–2440.

Confusing and conflicting information regarding the mechanisms of adjuvanticity of cholera toxin (CT)3 and Escherichia coli heat-labile toxin (LT) has recently been presented (1–8). These structurally closely related and well defined molecules consist of a single enzymatically active A1 subunit linked to a pentamere of cell membrane binding B subunits via a connecting A2 subunit (9–11). Whereas some investigators find intact adjuvanticity in mutants devoid of the ADP-ribosyltransferase activity, others have provided evidence suggesting that the enzymatic function is critically important (3, 4, 12). Thus, the role of the A1 subunit for the adjuvant effect is poorly understood. In fact, mutant molecules with single amino acid changes in the A1 subunit, rendering the enzyme partially or even completely inactive, have demonstrated adjuvant function comparable with that of the intact holotoxin (2, 3, 6, 7, 13). Therefore, it is not clear whether it is the ADP-ribosylating ability of the A1 subunit in the holotoxin or its structural role in the AB5 complex or both that is mediating the adjuvant effect.

Our laboratory achieved a major breakthrough in research on immunomodulation and vaccine adjuvant design when we constructed a gene fusion protein that combined the enzymatic activity of the A1 subunit of CT with a B cell targeting moiety derived from an Ig-binding fragment (D) of Staphylococcus aureus protein A. The novel immunomodulator, CTA1-DD, was found to exert adjuvant activity comparable with that of the intact CT after systemic as well as mucosal immunizations (14). However, in contrast to CT, and as a consequence of the lack of receptor-binding B subunits, the CTA1-DD adjuvant was found to be completely nontoxic in vitro and in vivo. Thus, we had successfully separated the adjuvant function from the toxic side effects of the cholera holotoxin in this novel fusion protein. Furthermore, the CTA1-DD molecule not only represents a promising new strategy for vaccine adjuvant design but also proves the concept that novel immunomodulators can be constructed as gene fusion proteins that target powerful bacterial enzymes to selected groups of cells, thereby avoiding harmful side effects. We believe that among many diverse applications, this strategy has an obvious applicability in the treatment of autoimmune diseases as well as in future vaccine development.

CT and LT are among the most powerful immunoenhancing molecules we know of today, and their use in vaccines is currently being exploited (8, 15). Since CT and LT have been intensely studied for several decades, our information on the structure and function of these toxins is extensive. For example, it is well documented that both CT and LT enter the cell via the ganglioside Gm1-receptor, present on most nucleated mammalian cells, and that this binding appears to be central for immunogenicity as well as adjuvanticity (9, 16–18). Furthermore, the toxins act via the A1 subunits to ADP-ribosylate GTP-binding proteins, of which the Gsa is thought to be most important, resulting in activation of adenylate cyclase and the subsequent increase in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) (19, 20). In addition, the toxins interact with yet other GTP-binding proteins known as

---

3 Abbreviations used in this paper: CT, cholera toxin; LT, Escherichia coli heat-labile toxin; ARFs, ADP-ribosylation factors; KLH, keyhole limpet hemocyanin; cAMP, cyclic adenosine 3′,5′-monophosphate; i.n., intranasal.

Copyright © 1999 by The American Association of Immunologists
Materials and Methods

DNA constructions

A DNA fragment consisting of 582 bp-encoding amino acids 1–194 of CTA1 (24) with flanking HindIII and BamHI sites was cloned into the pUC19 vector to generate pUC19-CTA1. This vector was used as a template for site-directed mutagenesis and subcloning of PCR products before sequencing. PCR was performed for mutagenesis of R7K in CTA1 with 5'-TTACGCCAACCTTCAATGATGATAAGTTATCTTAAGCCGAT TCGGA-3' and 5'-TTGATGATGTTTTAAAAGTTGATATCCA-3' as forward and reverse primers, respectively. The CTA1 mutations D109A and E112K were constructed using the following oligonucleotides; D109A, 5'-ACACTCCATATTTTGGGAGTATGGAATCCCACCTAAAGCAGAAACTTTTTG (forward), and E112K, 5'-GATACCATCCAACAGGAAACAGCTATGAC-3' (reverse) and 5'-ACCATCCATATTTTGGGAGTATGGAATCCCACCTAAAGCAGAAACTTTTTG (reverse) (forward) and 5'-ACA GGAAACAGCTATGAC-3' (reverse). Protein concentrations were determined by the Protein Assay (Bio-Rad).

Expression and purification of fusion proteins

The CTA1-DD, CTA1-R7K-DD, CTA1-D109A-DD, and CTA1-D112K-DD fusion proteins were produced in E. coli and affinity purified on an IgG column as described previously (14). The CTA1-DDhis and CTA1-DD34A/K38Ahis fusion proteins were expressed (14) and purified under reducing conditions (6 M guanidine·HCl). Denatured CTA1-DDhis and CTA1-DD34A/K38Ahis were recovered in the soluble fraction and subsequently purified using a nickel chelate column according to the manufacturer’s instructions (Novagen, Madison, WI). Purified CTA1-DDhis and CTA1-DD34A/K38Ahis were renatured by two sequential dialysis steps, overnight dialysis at 4°C against 1 M guanidine·HCl followed by 8 h of dialysis against 0.2 M acetic acid, pH 3. Typical yield per culture was 8–16 mg of fusion protein. The endotoxin content was <5 ng of LPS/μg of protein. The refolded soluble material was stored in 0.2 M acetic acid at 4°C.

Mice

Female C57BL/6 mice, age 8–12 wk, from B & K Universal AB, Sollentuna, Sweden were used for immunizations and nahru mice on the C57BL/6 background (Bomholt, Bomholtsgard, Denmark) were used for all B cell experiments in vitro.

Evaluation of adjuvanticity

Priming and booster immunizations were performed i.p. with purified CT (List Biological Laboratories, Campbell, CA) used at 1–2 μg/dose or affinity-purified CTA1-DD, CTA1-R7K-DD, CTA1-D109A-DD, CTA1-E112K-DD gene fusion proteins used at 10 μg/dose. The CTA1-DDhis and CTA1-DD34A/K38Ahis fusion proteins were used at a low dose which was equivalent to 5 μg and a higher dose equivalent to 10 μg of native CTA1-DD ADP-ribosyltransferase activity. As test Ags we used keyhole limpet hemocyanin (KLH; Calbiochem, San Diego, CA) or OVA (Sigma, St. Louis, MO), at 5 and 100 μg/dose, respectively. Putative adjuvants were admixed with Ag probe Ags in PBS, and five mice per group were immunized at 10-day intervals, and the mice were sacrificed 6–8 days after the final immunization. Adjuvanticity was evaluated as enhanced serum anti-KLH, anti-OVA, or anti-CTA (List Biologicals) log10 titers by ELISA essentially as described (14, 28).

Analysis of structure, binding, and conformation

A MiniProtein (Bio-Rad, Richmond, CA) system was used for SDS-PAGE analysis according to the manufacturer’s instructions. Ten micrograms of CTA1-DD, CTA1-R7K-DD CTA1-D109A-DD, CTA1-E112K-DD, CTA1-DDhis, and CTA1-DD34A/K38Ahis were dissolved in buffer and analyzed on a 12% Novex gel (San Diego, CA) under reducing conditions as described earlier (14). Protein concentrations were determined by the Bio-Rad DC Protein Assay (Bio-Rad).

The binding ability to mouse Ig of the CTA1-DD, CTA1-R7K-DD, CTA1-D109A-DD, CTA1-E112K-DD, CTA1-DDhis, or CTA1-DD34A/K38Ahis fusion proteins was assessed by ELISA. Briefly, polystyrene microtiter plates (Maxisorp Immunoplates; Nunc, Roskilde, Denmark) were coated with 10 μg/ml of murine IgG or IgM (PharMingen, San Diego, CA) or IgG Fab or IgG Fc fragments (Jackson ImmunoResearch Labs, West Port, PA) in PBS. Plates were blocked with 0.1% BSA in PBS/0.05% Tween 20 (PBST) for 1 h at 37°C, and serial 1:2 dilutions of the fusion proteins starting with 100 μg/ml were added to duplicate wells. Plates were incubated for 1 h at room temperature (temperature), washed, and subsequently reacted with alkaline phosphatase-conjugated chicken anti-staphylococcal protein A Abs (Innate System AB, Stockholm, Sweden), was added to the plates at 1/500 dilution. After 1h bound Abs were visualized using N-p-nitrophenylphosphate (Sigma) substrate in diethanolamine buffer, and the reaction was read at 405 nm using a Thermomax microplate reader (Mo- lonic Technologies, Sunnyvale, CA). The ability of mutant or native CTA1-DD molecules to bind to naïve B cells in vivo was assessed by FACS. Before analysis, i.v. injections of 20 μg of mutant or native CTA1-DD fusion protein were performed. CTA1-DD on B cells was detected by double-labeling of the isolated splenocytes with phycoerythrin and FITC-conjugated anti-IgD (PharMingen) and anti-protein A (Immun- system AB) Abs, respectively, using a FACScan (Becton Dickinson, San Jose, CA) (14). Gates were set on IgD+ cells which were analyzed for mean fluorescence intensity using histogram representation (14).
CTA1-specific Abs from CT-hyperimmunized mice (generated by three i.p. immunizations with CT at 2 μg/dose) were used to detect conformational changes of CTA1 in the mutant fusion proteins. Briefly, microtiter plates (Maxisorp Immuno plates; Nunc) were coated with 10 μg/ml of human IgG (Jackson ImmunoResearch Labs) followed by CTA1-DD or mutant proteins incubated at 100 μg/ml for 1 h at room temperature. Thereafter, hyperimmune CT-specific serum in serial dilutions was added to the plates and allowed to incubate for 1 h at room temperature. The degree of recognition of CTA1 in the different mutants by CTA-hyperimmune sera was visualized by the addition of alkaline phosphatase-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) at 1/200 followed by N-p-nitrophenylphosphate substrate as described above.

**ADP-ribosyltransferase and cAMP activity**

Determinations of ADP-ribosyltransferase and cAMP activity was performed as previously described (10, 14, 29). Briefly, the NAD:agmatine assay was used to detect enzymatic activity in twofold dilutions, beginning at 10 μg/ml of CTA1-DD, CTA1-R7K-DD, CTA1-D109A-DD, CTA1-E112K-DD, CTA1-DD, CTA1-DDhis, and CTA1-DDK4A/K384Ala or CT by assessing the ADP-ribosylagmatine formation through incorporation of [U-14C]adenine. A commercial cAMP-test kit was used according to the manufacturer’s instructions (Amersham International, Little Chalfont, U.K.). Briefly, spleen B cells (10^7 cells/ml) from natal mice or enriched peritoneal macrophages (4 × 10^6 cells/ml) were incubated with or without various concentrations of CT, rCTB CTA1-DD, or the various mutants and the level of cAMP induction was determined after 1 h or at later time points and calculated from a standard curve.

cAMP-independent B cell proliferation was determined essentially as described (30). Briefly, triplicate cultures of natal B cells (10^7 cells/well) were incubated in Iscove’s medium containing 10% FCS with 0.1 μg/ml CT, 10 μg/ml CTA1-DD, 10 μg/ml CTA1-R7K-DD, or 10 μg/ml rCTB together with 1 μM ionomycin with or without 10 mM 3-aminobenzamide (Sigma-Aldrich, St. Louis, MO) (31). After 3 days, [1H]thymidine (1 μCi; Amersham) was added for the final 18 h of culture. The cells were harvested on glass fiber filters, and the [3H]TdR uptake was assessed using a β-scintillation counter (1450 MicroBetaTriLux, Wallac, FL).

**Statistical analysis.** We used the t test for independent samples for analysis of significance.

**RESULTS**

*The ADP-ribosyltransferase activity of CTA1-DD is critical for the adjuvant function*

The CTA1-DD fusion protein provides a unique possibility to experimentally address the role of the enzymatic activity of CTA1 for the adjuvant effect of the bacterial enterotoxins, CT and LT (14). A broad approach was taken to evaluate whether the CTA1-DD molecule required ADP-ribosyltransferase activity or not for the adjuvant function. We constructed three enzymatically inactive mutants, CTA1-R7K-DD, CTA1-D109A-DD, and CTA1-E112K-DD (Fig. 1). Using the cell-free NAD:agmatine assay, we observed that while the mutants failed to demonstrate ADP-ribosyltransferase activity even at the highest doses tested (Fig. 2), CTA1-DD exhibited an enzymatic activity that was roughly 50%, not only abolish enzyme function but also result in structural changes of the molecule. However, as illustrated by the representative data with the CTA1-R7K-DD mutant in Fig. 4, we were unable to disclose any conformational or degradative changes in the enzymatically inactive mutants: Mutants as well as the native CTA1-DD molecule all behaved as monomeric fusion proteins 37 kDa in mass (Fig. 4A). CTA1-specific antiserum from CT-hyperimmunized mice recognized CTA1-DD and the mutants equally well (Fig. 4B). Binding of the mutants to mouse IgD^+^ B cells after i.v. injection (Fig. 4C), or to intact IgM or IgG, or to IgG Fc and Fab fragments in solid phase in ELISA plates (Fig. 5) was retained.

![FIGURE 1. Schematic drawing of the CTA1 subunit and the amino acid substitutions in the enzymatically active cleft that were tested in the study.](http://www.jimmunol.org/)

![FIGURE 2. ADP-ribosyltransferase activity. The NAD-agonist assay was used to detect enzymatic activity of serial 1/2-dilutions of CT ( ), CTA1-DD ( ), CTA1-R7K-DD, CTA1-D109A-DD or CTA1-E112K-DD ( ) were assayed for ADP-ribosylagmatine formation through incorporation of [U-14C]adenine. The values represent mean counts per minute (cpm) of three experiments with SD <5%. The mean background activity was 1987 cpm.](http://www.jimmunol.org/)
These results are in agreement with unaltered structural and Ig-binding properties of the mutant CTA1-DD fusion proteins.

In addition, we used CTA1-specific serum Abs as an internal standard to assess altered immunoenhancing activity of the mutated CTA1-DD molecules in vivo. CTA1-specific serum Abs were determined after i.p. immunizations. Both CT and CTA1-DD exerted strong immunoenhancing effect on CTA-specific responses, whereas, by contrast, the mutated CTA1-DD, represented by the CTA1-R7K-DD and CTA1-E112K-DD mutations in Fig. 6, stimulated poor or no (p < 0.05) CTA1-specific serum Ab titers. Thus, the lack of enzymatic activity dramatically reduced the immunogenicity of the CTA1-moiety itself.

**Adjuvanticity of CTA1-DD is dependent on the ability to bind to Ig**

Next we investigated the importance of the Ig-binding ability of the DD dimer for the adjuvant function of CTA1-DD. On the basis of earlier findings, we constructed a fusion protein that contained two mutations in the D fragments: Ile34 to Ala and Lys38 to Ala, CTA1-DDI34A/K38Ahis mutant (25, 26, 32). To enable purification of the non-Ig-binding fusion proteins, we added a C-terminal histag consisting of six histidine residues (His), to the construct, and purification was performed using a nickel chelate column. For comparison, we also constructed a fully competent Ig-binding CTA1-DDhis fusion protein that was similarly purified. After assessment of the ADP-ribosylating capacity of the various constructs, we compared their immunoenhancing ability using doses of equivalent enzymatic activity. We found that the CTA1-DDI34A/K38Ahis mutant exhibited significantly (p < 0.01) lower immunoenhancement of KLH-specific serum Ab responses than did the native Ig-binding CTA1-DDhis fusion protein (Fig. 7). With a low dose of CTA1-DDI34A/K38Ahis mutant, no adjuvant effect was achieved, while the Ig-binding CTA1-DDhis gave substantial enhancement. With a higher dose, the immunoenhancing
The adjuvant effect of CTA1-DD appears to be cAMP independent

In the present study, we have shown that the adjuvant effect of CTA1-DD was comparable with that of CT and required the ADP-ribosyltransferase activity, as exemplified by the lack of adjuvanticity of, e.g., the CTA1-R7K-DD mutation. Since CT acts by ADP-ribosylating G-proteins, primarily the Gsα, that activate adenylate cyclase, leading to increased intracellular cAMP levels, we investigated the effects of CTA1-DD on intracellular cAMP levels. Unexpectedly, we found that the intracellular cAMP levels in freshly isolated B cells or macrophages after treatment for 1 h with CTA1-DD did not change relative to control cultures, while they were substantially increased, in a dose-dependent manner, after treatment with CT (Table I). The CTA1-DD fusion protein did not affect cAMP levels even in doses 10,000-fold in excess of the...
active dose of CT (Table I), nor did we observe increases in intracellular cAMP at later times, whereas CT promoted a dose-dependent increase of intracellular cAMP at 1, 2 4, 8, or 21 h (not shown). On the other hand, as expected, the CTA1-R7K-DD mutant or rCTB both failed to affect intracellular cAMP levels (Table I). Consequently, the mechanism by which CTA1-DD exerts its immunoenhancing effect appears to be independent of cAMP and may not involve Gs, which is believed to be the main target for the enzymatic activity of CTA1 in the intact holotoxin.

Induction of B cell proliferation confirms that CTA1-DD exerts ADP-ribosyltransferase activity on intact cells

Although CTA1-DD adjuvant was shown to act through ADP-ribosylation of target proteins in the cell-free NAD-agmatine system (Fig. 2), we did not know whether the fusion protein indeed also could act on intact cells. To this end, we took advantage of a previous study demonstrating that CT could exert cAMP-independent stimulatory effects on B cell proliferation in the presence of ionomycin (30, 33). As illustrated in Fig. 9, both CT and CTA1-DD, but not rCTB or CTA1-R7K-DD, stimulated significant B cell proliferation in the presence of ionomycin (Fig. 9). The effect of CTA1-DD was dramatic and dose dependent, while that of CT was more modest, even in higher doses (not shown). Neither the CTA1-R7K-DD mutant nor rCTB could significantly support ionomycin-driven B cell proliferation. Moreover, when we added 3-aminobenzamide, a blocker of ADP-ribosylation (31), the CTA1-DD- and CT-stimulated B cell proliferation was effectively inhibited to near background levels, while no effect of 3-aminobenzamide was seen on B cell proliferation in the presence of the CTA1-R7K-DD mutant or rCTB (Fig. 9). These results unequivocally demonstrated that the CTA1-DD fusion protein acts on intact B cells by exerting ADP-ribosyltransferase activity.

**FIGURE 8.** The CTA1-DDI34A/K38Ahis mutant lacks Fe-binding ability but has partly retained the Fab-binding ability to mouse IgG. The ability of CTA1-DD with mutations in the D fragments to bind to mouse IgG in solid phase was investigated by ELISA. The CTA1-DDhis and CTA1-DDI34A/K38Ahis were analyzed in duplicates, and the results represent the mean results of three experiments with SD of <5%.

**FIGURE 9.** CTA1-DD acts on intact B cells as assessed by the ability to promote ionomycin-driven ADP-ribosyltransferase-dependent proliferation. Enriched splenic B cells from nu/nu mice were incubated (2 \times 10^5 cells/well) in Iscove’s total medium containing 10% FCS with 0.1 μg/ml CT, medium and 1.0 μg/ml rCTB, CTA1-DD, or CTA1-R7K-DD, together with 1 μM ionomycin (black bars) or with 1 μM ionomycin and 10 mM 3-aminobenzamide (striped bars), a blocker of ADP-ribosyltransferase activity. B cell proliferation was assessed by ^3H^thymidine uptake on the third day of incubation using a β-scintillation counter. Data are mean counts/minute (cpm) ± SD of triplicate cultures and representative of three independent experiments. Values for cultures with CT or CTA1-DD are significantly different from those with the enzymatically inactive mutant, CTA1-R7K-DD, rCTB, or medium (asterisk in oval) or those obtained in the presence of 3-aminobenzamide (p < 0.05) (asterisk).

<table>
<thead>
<tr>
<th>Immunomodulator</th>
<th>B Cells</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
<tr>
<td>CTB</td>
<td>8.9</td>
<td>14.2</td>
</tr>
<tr>
<td>CTA1-DD</td>
<td>4.4</td>
<td>3.6</td>
</tr>
<tr>
<td>CTA1-R7K-DD</td>
<td>1.9</td>
<td>3.1</td>
</tr>
<tr>
<td>r-CTB</td>
<td>2.6</td>
<td>3.2</td>
</tr>
<tr>
<td>CTA1-DD</td>
<td>0.1</td>
<td>3.0</td>
</tr>
<tr>
<td>CTA1-R7K-DD</td>
<td>0.01</td>
<td>2.6</td>
</tr>
<tr>
<td>Medium</td>
<td>2.5</td>
<td>3.4</td>
</tr>
</tbody>
</table>

* A dose-response analysis of induction of intracellular cAMP was performed with spleen B cells from nu/nu mice or enriched peritoneal macrophages (PMF) from C57BL/6 mice. Cells were incubated for 1h in the presence or absence of indicated concentrations of CT, r-CTB, CTA1-DD, or CTA1-R7K-DD. cAMP values are expressed in picomols per 10^7 B cells or 4 \times 10^6 PMF and are representative of seven experiments giving similar results. rCTB, recombinant CTB subunit; NT, not tested.
Discussion

The mechanism for the adjuvant effect of the bacterial ADP-ribosylating enterotoxins, CT and LT, is still poorly understood (8, 34). A current controversy in the field is the degree of adjuvanticity contributed by the G_{41} receptor-binding B subunits relative to that contributed by the enzymatically active A1 subunit (8, 35, 36). Here we show, using our newly constructed CTA1-DD fusion protein (14), that ADP-ribosyltransferase activity of the CTA1 is a prerequisite for the adjuvant function. Moreover, adjuvanticity of the fusion protein is dependent on its ability to bind to Ig via the DD dimer. Thus, contrary to recently published work from several laboratories using mutant holotoxins, we can unequivocally demonstrate that the ADP-ribosyltransferase active A1 subunit has potent immunomodulating ability of its own (1–3, 13). By excluding the B subunits and via direct targeting of the full enzymatic activity of the CTA1 subunit to B cells, and perhaps other APC, we have achieved full adjuvanticity with no toxicity. We believe that the CTA1-DD fusion protein, therefore, represents an important advancement in immunomodulation and vaccine adjuvant research.

The results of the present study are in keeping with findings reported by Rappuoli and coworkers (6, 7), who demonstrated that mutants of CT or LT holotoxin with partial ADP-ribosylating activity, CTS106 and LTR72, were significantly stronger adjuvants than enzymatically inactive mutants. However, the inactive mutants, CTK63 or LTK63, had significantly better adjuvant ability than enzymatically inactive mutants. A single amino acid substitution in the active cleft disrupted the possibility that the AB₅ complex may exert an adjuvant effect that is independent of the enzymatic activity but dependent on structural or binding properties of the A1 subunit. The authors put forward a hypothesis that adjuvanticity of the bacterial holotoxins is composed of two elements, enzymatic activity and, secondly, nontoxic AB₅ complex-associated effects (7). Possible targets for this second effect could be the cell membrane interaction of the B subunits or molecules that are bound by the A1 subunit. Because CTB and LTB are generally poor immunogens and adjuvants (6, 37–40) as compared with the nontoxic mutants, one might speculate that it is the interaction of the A1 subunit with some undefined cellular proteins that is the most interesting. It is known that the enzymatic activity of CT and LT is enhanced by the interaction of these proteins with intracellular ARFs, and it is conceivable that an enzymatically inactive mutant A1 subunit might structurally interact with these GTP-binding proteins (9, 21, 41). Although this hypothesis is attractive, our results using the CTA1-DD fusion protein do not support this explanation. In fact, we failed to demonstrate adjuvant activity in three different inactive CTA1-DD mutants. A single amino acid substitution in the active cleft disrupted the enzymatic activity but did not seem to alter the conformation or structure of the A1 subunit, thus probably still allowing binding to ARFs or other molecules. Thus, although our current results do not preclude a “structural” effect of CTA1, we believe our observations point in a different direction.

We would like to hypothesize that it is the ADP-ribosyltransferase activity of the A1 subunit per se that is important for the immunomodulating effect. Our study clearly has demonstrated that the enzymatic activity of the A1 subunit constitutes a unique and separate immunomodulating ability of the bacterial enterotoxin. The strength of this immunomodulation can easily be appreciated by the fact that we achieved an adjuvanticity with the CTA1-DD fusion protein of equal magnitude to that of the intact CT. The dose required for such an effect was roughly threefold higher than that of CT (14), supporting the idea that the holotoxins, indeed, also host additional nontoxic AB₅ complex-associated adjuvant properties. By inference, although not yet demonstrated, we predict that it would be possible to replace or omit the A1 subunit from the AB₅ complex and still achieve stronger adjuvanticity than that obtained with the B subunits alone. No documentation is available on the adjuvant role of the A2 subunit in the AB₅ complex. Perhaps a deletion of A1 would generate a molecule that still matches the AB₅ complex in adjuvant capability. Such a molecule has been found an efficient delivery system for mucosal immune responses to epitopes replacing the A1 subunit epitopes (42).

Thus, with regard to the second immunomodulating effect of the holotoxins mediated by the AB₅ complex, we firmly believe that it is separate from the enzymatic activity of the A1 subunit. The question is whether this effect is operating through a single or multiple mechanisms. If the AB₅ complex is more stable in vivo, as been suggested (43), or even if it has a broader spectrum of interactions with cell membrane or cytosolic substructures than the B subunits alone, it is probable that immunomodulation dependent on the B subunit.

Such a notion can explain why a great variability in adjuvanticity has been reported for the nontoxic mutants and the B subunits. In particular, the route of administration is critically influencing the degree of adjuvanticity. No doubt the oral route is most demanding, whereas intranasal (i.n.) immunization appears to be more permissive (6, 37). Indeed, several investigators have reported substantial adjuvant effects by CTB or LTB alone given i.n., whereas CTB or LTB, for the most part, has been found inactive given perorally as adjuvants (35, 40, 44, 45). Interestingly, Yamamoto et al. (3, 46) recently reported that the E112K mutant CT, which is the same substitution tested by us in the CTA1-E112K-DD fusion protein, gave enhancing effects on immune responses after peroral or s.c. administration similar to that observed with the intact CT. Currently, we have no explanation for this discrepancy, but in the present study we specifically used the i.p. route for immunization to avoid possible involvement of variability in adjuvant and Ag uptake, as would be expected with the peroral or i.n. routes. An earlier study from our laboratory also failed to show adjuvant function of LT-E112K after oral immunization, whereas de Haan et al. (1, 40) found that nasal administration of this mutant gave good adjuvant effects. Taken together, these observations indicate that the route of adjuvant administration and type of Ag used for evaluation are crucial elements to consider when assessing the immunoenhancing effects of the mutant holotoxins.

Nevertheless, in practical terms all AB₅ complex-dependent systems appear to have the same limitation, namely the requirement for uptake via the ganglioside G₄₁ receptor. Since this receptor is expressed on most nucleated cells, these adjuvant molecules can never be selective in their action. Although Douce et al. have, using the CTK63 and LTK63 mutants, provided evidence to suggest that LT may have broader immunogenic and adjuvant effects than CT, most investigators have found that both molecules lack these abilities in the mutant non-G₄₁ ganglioside-binding forms (16, 17, 36). This is despite the fact that LT, as opposed to CT, can bind several other carbohydrate ligands (47). Thus, adjuvanticity of CT and LT appears to depend on the ganglioside G₄₁ receptor pathway (16, 17). It should be mentioned, however, that at variance with these observations de Haan et al. (1) have reported a non-G₄₁-binding LT mutant, LT-G33D, as well as a mutant that lacks both binding and ADP-ribosyltransferase activity, LT-E112K/G33D, with retained adjuvant function after i.n. administration. This latter observation is controversial, but, together with the reports of strong adjuvant effects of the B subunits given i.n., it clearly demonstrates current problems in evaluation of adjuvant function using the i.n. route of administration.
The unique strength of the CTA1-DD system is that it targets the full enzymatic activity of the CTA1 to cells that are engaged in the formation of an immune response, leaving all other cells at rest. The inherent problem with the mutant holotoxins using the ganglioside G_{M1} pathway, incurred by the risk of toxicity, is the required balancing of adjuvanticity against enzymatic activity. Elegant studies have demonstrated that partially active LTR72 mutant can give similar adjuvant effects to the intact holotoxin in doses 50–100-fold higher (7). Such dose increases appeared to be safe and nontoxic in mice, but for clinical use, adjuvant active, but nontoxic, doses may be difficult to achieve. Ongoing clinical trials with some of these mutants will hopefully answer this critical question (48–50).

What is most remarkable with the CTA1-DD adjuvant, however, is the ability to circumvent the natural ganglioside G_{M1} pathway for entry of CTA1 into the cell via binding through the DD dimer. Although we have not completely defined the ligands for the DD dimer, we strongly believe that the B cell receptor is the most likely candidate (14). Previous and ongoing studies point to the idea that B cell targeting rather than binding to dendritic cells and macrophages occurs after injection (14). Whether this effect is responsible for the adjuvanticity or not to be arrived at, clearly, the fact that admixed CTA1-DD, without conjugation to the Ag, achieves strong immunoenhancement in CD4^{+} T cell priming and humoral immunity (14) argues in favor of an involvement in Ag presentation. We have also reported that up-regulation of the costimulatory molecule CD86 is a primary event after treatment of naive B cells with CTA1-DD (14).

In the present study, we extend the evidence for an immunomodulating effect of CTA-DD on binding to the B cell, by showing that B cell proliferation in the presence of ionomycin is greatly increased. This regard, the effect of CTA1-DD mimics that of CT, whereas rCTB or the enzymatically inactive mutant CTA1-DD failed to affect this system. Benzamide, a known inhibitor of ADP-ribosyltransferase activity, substantially abrogated the proliferation induced by CTA1-DD and CT, clearly indicating that these agents use ADP-ribosyltransferase activity for the effect (30). Although CTA1 in the holotoxin is thought to ADP-ribosylate the G_{s} protein, leading to an activation of adenylyl cyclase and subsequent increase of intracellular cAMP, we failed to show an effect on cAMP levels. In spite of numerous experiments using different conditions, high doses of the fusion protein and different time points, using isolated B cells or macrophages or two different murine B cell lines (X16, CH12LX), we were unable to mimic the effect of CT on cAMP levels. This observation was unexpected and highly surprising.

A speculation derived from the findings mentioned above is that CTA1 in the form of a fusion protein uses a pathway distinct from the classical G_{s}o-adenylate cyclase-cAMP pathway for its adjuvant effect. In fact, we believe our report is the first to suggest that CT exerts adjuvant activity independently of cAMP. Even though the pathway for entrance of CTA1 into the target cell differs between the fusion protein and the holotoxin, the present study indicates that a substrate (or substrates), which is ADP-ribosylated, in both cases is targeted by CTA1. The nature of this substrate may, thus, not be G_{s} protein but one or several other G-proteins, or alternatively, it may be that the G_{s} protein, in a different subcellular location, is not acting on adenylyl cyclase. Future studies in our laboratory will address these important and challenging questions.

Acknowledgments

We thank Karin Schön for skilled technical assistance and Martin Norin for help in preparing Fig. 1.

References


