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Novel Immunostimulatory Agent Based on CpG Oligodeoxynucleotide Linked to the Nontoxic B Subunit of Cholera Toxin

Jenni Adamsson,* Marianne Lindblad,* Annika Lundqvist,* Denise Kelly,† Jan Holmgren,* and Ali M. Harandi2*

In this study, we report the development of a novel, rationally designed immunostimulatory adjuvant based on chemical conjugation of CpG oligodeoxynucleotide (ODN) to the nontoxic B subunit of cholera toxin (CTB). We demonstrate that the immunostimulatory effects of CpG can be dramatically enhanced by conjugation to CTB. Thus, CpG ODN linked to CTB (CTB-CpG) was shown to be a more potent stimulator of proinflammatory cytokine and chemokine responses in murine splenocytes and human PBMCs than those of CpG ODN alone in vitro. The presence of CpG motif, but not modified phosphorothioate ODN backbone, was found to be critical for the enhanced immunostimulatory effects of CTB-CpG. Our mode-of-action studies, including studies on cells from specifically gene knockout mice suggest that similar to CpG, CTB-CpG exerts its immunostimulatory effects through a TLR9/MyD88- and NF-κB-associated pathway. Surprisingly, and as opposed to CpG ODN, CTB-CpG-induced immunity was shown to be independent of endosomal acidification and resistant to inhibitory ODN. Furthermore, preincubation of CTB-CpG with GM1 ganglioside reduced the immunostimulatory effects of CTB-CpG to those of CpG ODN alone. Interestingly, conjugation of CpG ODN to CTB confers an enhanced cross-species activity to CpG ODN. Furthermore, using tetanus toxoid as a vaccine Ag for s.c. immunization, CTB-CpG markedly enhanced the Ag-specific IgG Ab response and altered the specific pattern of Ab isotypes toward a Th1 type response. To our knowledge, CTB is the first nontoxic derivative of microbial toxins discovered that when chemically linked to CpG remarkably augments the CpG-mediated immune responses. The Journal of Immunology, 2006, 176: 4902–4913.

Unmethylated CpG dinucleotides with particular sequence context (CpG motifs) occur at high frequency in microbial DNA and stimulate the mammalian immune system (1). Cellular responses to CpG DNA require initial internalization of the DNA followed by endosomal acidification. CpG binds to TLR9 located intracellularly on endosomal membranes. TLR9 signaling proceeds through recruitment of MyD88 leading to activation of several kinases, including IL-1R-associated kinase and TNFR-associated factor 6. The signal cascade culminates in the activation of NF-κB, AP-1, and members of the MAPK family that trigger rapid induction of various proinflammatory and Th1-polarizing cytokines and chemokines, up-regulation of costimulatory molecules on APCs, and activation of B cells for proliferation, IL-6 secretion, and Ab production (2). The immunostimulatory features of bacterial DNA can be mimicked by synthetic oligodeoxynucleotide (ODN)3-containing CpG motifs. The use of CpG ODN as a Th1-tilting immunostimulator/adjuvant either singly or in combination with various Ags for induction of both systemic and mucosal immunity in experimental animals has been documented (2, 3).

The immunostimulatory effects of CpG ODNs are dependent on a number of factors, including DNA sequence, nature of the backbone, and the presence of species-specific motifs. Although phosphodiester (PO) CpG ODNs are poor inducers of immune responses, CpG ODNs with a modified phosphorothioate (PS) backbone possess potent immunostimulatory effects (2, 4). Mice and humans respond preferentially to different CpG motifs. In mice, the optimal motif consists of a central unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines such as GACGTT (e.g., CpG ODN 1826) (5, 6). For humans, GTCGTT in multiple copies, separated by TT dinucleotides (e.g., CpG ODN 2006) is optimal (7).

Efforts have been made to augment the immunostimulatory effects of CpG ODNs. Thus, adsorption of CpG ODN to the surface of cationic poly(3,3′-lactide-co-glycolide) microspheres was shown to induce enhanced secretion of IFN-γ and IFN-α from human PBMC compared with CpG ODN alone (8). Furthermore, a synergistic adjuvant activity was reported when CpG ODN was mixed with cholera toxin (CT) or Escherichia coli heat-labile enterotoxin (LT); however, a mixture of CpG ODN and nontoxic derivatives of CT and LT, i.e., the B subunit of CT (CTB) and the mutant of LT

Abbreviations used in this paper: ODN, oligodeoxynucleotide; PO, phosphodiester; PS, phosphorothioate; CT, cholera toxin; LT, Escherichia coli heat-labile enterotoxin; CTB, B subunit of CT; TGN, trans-Golgi network; ER, endoplasmic reticulum; TT, tetanus toxoid; EU, endotoxin unit; WMN, wortmannin.
protein derivative LTK63, had no synergistic effects on the magnitude of the immune response (9–11).

CT is an AB5 hexameric protein responsible for the severe cholera diarrhea caused by *Vibrio cholera* (12). CT consists of five identical B subunits and a single A subunit. Cell recognition and binding of CT are conducted by the B pentamer via specific binding to the ganglioside GM1 receptor, a glycosphingolipid found ubiquitously on the surface of mammalian cells. High-affinity interaction of CTB with GM1 ganglioside receptors in lipid rafts of the plasma membrane triggers holotoxin internalization into smooth endocytic vesicles that are targeted to the trans-Golgi network (TGN) and the endoplasmic reticulum (ER). The enzymatically active A subunit is then directed to the interior of the target cell where it causes an increase in the intracellular level of cAMP (13). Several reports indicate that the immunogenicity of protein Ags can be enhanced when they are conjugated to CTB rather than simply mixed with it. CTB, as opposed to CT, can be safely administered to humans (14).

In this study, we investigated the possibility of enhancing immunostimulatory and adjuvant effects of CpG by combining the immunostimulatory effect of Cpg with the specific cell binding and intracellular shuttling ability of CTB. To achieve this goal, we chemically linked synthetic CpG ODN to rCTB protein. We report here that the immunostimulatory effects of CpG can be dramatically potentiated by conjugation to CTB. Thus, Cpg linked to, but not simply admixed with, CTB is a more potent stimulator of cytokine and chemokine responses in murine splenocytes and human PBMCs than CpG ODN in vitro. Interestingly, conjugation of PO CpG ODN with little immunostimulatory effect to CTB converts it into a potent stimulator of cytokine and chemokine responses in vitro. We also report a series of studies that analyze the mode of action of CTB-CpG. These studies suggest that similar to Cpg, CTB-CpG exerts its immunostimulatory effects through a TLR9/MyD88- and NF-κB-dependent pathway. Surprisingly and as opposed to Cpg ODN alone, CTB-CpG-induced immunity was independent of endosomal acidification and also resistant to inhibitory ODN. Preincubation of CTB-CpG with GM1 ganglioside reduced the immunostimulatory effects of CTB-CpG to those of CpG ODN alone. Furthermore, we could show that the conjugation of CpG ODN to CTB can overcome the species-specificity of CpG ODN. Finally, using tetanus toxoid (TT) as a vaccine Ag for s.c. immunization of mice, CTB-CpG has been shown to markedly augment the induction of Ag-specific IgG Ab response and to tilt the specific pattern of Ab isotypes toward a Th1 type response, suggesting that CTB-CpG could be used as a potent immune adjuvant. To our knowledge, CTB is the first nontoxic derivative of microbial toxins discovered that when chemically linked to CpG dramatically augments the CpG-mediated immune responses. This observation warrants further exploration of the impact of the new class of immunostimulator/adjuvant based on CpG ODN coupled to CTB for inducing more potent immune responses.

Materials and Methods

**ODN and reagents**

Cpg ODN 2006, a 24-mer that contains four copies of a CpG motif (TCG TCG TTT TGT CCA TTG GAT TGT), was used for human in vitro studies. Human ODN IMTO22, a 24-mer with no CpG motifs (TGC TGG AAA AGA CCA AAA GAG CAA), was used as a control. For mouse studies, Cpg ODN 1826, a 20-mer containing two copies of a CpG motif (TCC AGC CAC TCT TCC AGC TT), was used as a control. Mouse ODN 1502, a 20-mer, which contains two copies of a GpC motif (GpC ODN) (GAG CAA GCT GGA CCT TCC AT), was also used. All ODNs were used with complete PS backbone. Cpg 2006 was used with either a PS or a PO backbone. The ODNs were purchased from Cybergene (Novum Research Park) and Operon. ODNs were tested for endotoxin, and the LPS contents were <0.03 endotoxin units (EU)/ml.

rCTB protein and TT were provided by SBL Vaccines. All other reagents were purchased from Sigma–Aldrich unless stated otherwise.

**Conjugation of CpG ODN to CTB**

The conjugation of CpG ODN to CTB was performed by mixing thiolated ODN with maleimide-activated CTB at room temperature overnight, followed by purification by gel filtration through Superdex 200 (Amersham Biosciences). The high molecular peak was collected and protein concentration was determined using a protein assay (micro BCA; Pierce), with CTB as a reference protein. Concentration of nucleic acid was determined by measuring the absorbance at 260 nm. The CTB-ODN conjugates were analyzed by SDS-PAGE and CTB and CpG ODN in the conjugate were visualized with Coomassie blue (Sigma–Aldrich) and SYBR Green (Molecular Probes), respectively. The GM1-binding activity of CTB-ODN conjugates was confirmed by a solid-phase GM1 ELISA (15). The LPS contents of ODN-CTB conjugates were 0.1 EU/ml.

**Animals**

Six- to 10-wk-old mice were used for all experiments. C57BL/6 mice were purchased from M&B. C3H/HeN and C3H/HjE mice were purchased from Charles River Laboratories. MyD88 (16) and TLR9 (17) mice on C57BL/6 background were originally from Prof. S. Akira’s laboratory (gift from Prof. M. J. Wick, Department of Clinical Immunology, Gothenburg University, Göteborg, Sweden). Animals were housed in microisolators under specific pathogen-free conditions at the Experimental BioMedicine animal facility (Sahlgrenska Academy, Göteborg University, Göteborg, Sweden). All experiments were performed with the approval from the Ethical Committee for Laboratory Animals (Göteborg, Sweden).

**In vitro human proliferation assays**

Human PBMC were isolated from buffy coats provided by the blood bank of the Sahlgrenska Hospital (Göteborg, Sweden). PBMCs were prepared from buffy coats by Ficoll-Hypaque (Amersham Biosciences) density gradient centrifugation. PBMCs were suspended in basal Iscove’s medium (Biochrom) supplemented with 2 mM l-glutamine (VWR), 50 μM 2-ME, 100 μg/ml gentamicin, and 10% heat-inactivated FCS (referred to as Iscove’s complete medium) and seeded in triplicates in 96-well flat-bottom plates (Nunc) and stimulated with different concentrations of ODNs, recombiant CTB, CTB + CpG, CTB-ODNs. Supernatants were collected at different time points and assayed for cytokines and chemokines contents.

**In vitro mouse proliferation assays**

Spleens were removed from anesthetized animals under asptic conditions and homogenized. Splenocytes were resuspended in the Iscove’s complete medium, and incubated in the presence of CpG ODN, rCTB, a mixture of CTB and CpG or CTB-CpG conjugate. Supernatants were collected and examined for their contents of cytokines and chemokines.

For mode of action experiments, cell suspensions of murine splenocytes were pretreated with chloroquine (2 or 4 μg/ml), quinacrine (0.1 or 0.4 μg/ml), wortmannin (2.5 μg/ml), or gliotoxin (0.25 μg/ml) for 2 h at 37°C, followed by cultivation in the presence of CpG ODN (0.3 μg/ml), CTB, CTB + CpG or CTB-CpG. In some experiments, inhibitory ODN 1502 (1, 3, 9 μg/ml) or polymyxin B (10 μg/ml) was added to the cell culture at the same time as reagents.

For the GM1 receptor blocking experiment, the reagents were preincubated with GM1 ganglioside (2.5 or 10 μg/ml) for 30 min at 37°C before being added to the C57BL/6 splenocyte culture.

**Cytokine and chemokine quantification**

Concentrations of cytokines and chemokines in pooled culture supernatants were analyzed using cytokine/chemokine DuoSet, fluorokine ELISA, or matched Ab pairs (R&D Systems), according to the manufacturer’s recommendations.

**NF-κB EMSA**

Human PBMCs were stimulated with CpG ODN, CTB, or CpG-CTB for periods up to 2 h. Following stimulation, cells were harvested into ice-cold PBS and lysed in hypotonic buffer (0.125% Igepal CA-630, 5 mM HEPES (pH 7.9), 10 mM KCL, 1.5 mM MgCl2). Following centrifugation...
(13,000 \times g, 1 \text{ min, } 4°C), nuclear pellets were resuspended in hypertonic lysis buffer (0.25% Igepal CA-630, 5 mM HEPES (pH 7.9), 25% glycerol, 500 mM MgCl₂, 0.2 mM EDTA) for 2 h at 4°C with constant mixing. Nuclear extract supernatants were recovered by centrifugation (13,000 \times g, 10 \text{ min, } 4°C), aliquoted and stored at 80°C before EMSAs. NF-κB DNA-binding activities of nuclear extracts were determined using 4 μg of nuclear protein incubated with [γ-32P]ATP NF-κB double-stranded consensus oligonucleotide (Promega). For supershift analysis, 1 μg of p65 (Rel A) Ab (Santa Cruz Biotechnology) was added to the nuclear extract mix. Samples were resolved using 5% (w/v) nondenaturing polyacrylamide gels. Gels were dried and NF-κB DNA-binding protein complexes were visualized using a Fujifilm LAS-1000 image analyzer.

**Ab measurements**

Low-binding 96-well plates (Nunc) were coated with TT at a concentration of 2 μg/ml in carbonate buffer at 4°C overnight. Plates were blocked with 4% milk (Semper) for 2 h at 37°C. Bound Abs were detected using HRP-conjugated goat-anti-mouse IgG, IgG1, or IgG2c (Jackson Immunoresearch Laboratories). The plates were developed with o-phenylene-diamine dihydrochloride and read at 450 nm. The sample IgG titer was defined as the sample dilution giving an OD value of 0.4 above the background value.

**Results**

**CTB-CpG as a potent stimulator of cytokines and chemokines in human PBMCs**

We sought to examine whether conjugation of CpG ODN to CTB would enhance immunostimulatory effects of CpG ODN in human PBMCs. To this end, human PBMCs were cultured in the presence of increasing concentrations of CTB or CpG 2006, either alone or in mixed (CTB + CpG 2006) or conjugated (CTB-CpG 2006) form. Supernatants of the cells were collected at different time points and examined for proinflammatory cytokines and chemokines.

Basal levels of MIP-1α and MIP-1β were observed in the supernatants of the human PBMCs at 24 h (1350 ± 400 and 9779 ± 929 pg/ml/million cells, respectively). Incubation of the cells with CTB did not induce any MIP-1α or MIP-1β productions over those of untreated cells at any concentrations or time points examined (Fig. 1, A–C). Incubations of PBMCs with CpG 2006 (3 μg/ml) induced a 3- and 5-fold increases in MIP-1α and MIP-1β productions over those of cells alone at 24 h, respectively, that declined to the basal level within 48 h. No additional increase was observed in the supernatants of the cells treated with CTB + CpG 2006. However, CTB-CpG 2006 (3 μg/ml) induced robust, dose-dependent induction of MIP-1α (182-fold increase) and MIP-1β (46-fold increase) responses at 24 h and remained elevated for 72 h (Fig. 1, A–F).

IL-6 production followed the same pattern as those observed for MIP-1α and MIP-1β. Thus, CpG 2006 and CTB + CpG 2006 induced comparable levels of IL-6 response in human PBMCs. CTB-CpG 2006, in contrast, induced a very strong IL-6 response in a dose-dependent manner that remained moderately high for 72 h (Fig. 1, G–I).

As tabulated in Table I, CpG 2006 (3 μg/ml) induced moderate levels of IL-1β (4-fold increase over untreated cells) and MCP-1 (2-fold increase over untreated cells) productions in human PBMCs. CpG 2006 did not induce any appreciable change in the level of IL-8 in human PBMCs. CTB-CpG 2006 induced relatively

![FIGURE 1. Comparison of CpG ODN, CTB, CTB + CpG, and CTB-CpG for their ability to induce MIP-1α, MIP-1β, and IL-6 responses in human PBMCs. A–I, Human PBMCs were incubated with different concentrations of CTB or CpG 2006, either alone or in the mixed or conjugated form. Cell supernatants were collected at 24 h (A, D, and G), 48 h (B, E, and H) and 72 h (C, F, and I) and examined for MIP-1α, MIP-1β, and IL-6 contents by ELISA. Data shown are representative of five independent experiments and are expressed as mean of duplicate ELISA samples ± SEM.](http://www.jimmunol.org/Downloadedfrom)
strong IL-1β (167-fold increase over CpG 2006), IL-8 (5-fold increase over CpG 2006), and MCP-1 (4-fold increase over CpG 2006) responses in human PBMCs (Table I). Neither CpG 2006 nor CTB-CpG 2006 elicited any appreciable change in the levels of IFN-inducible protein-10 and RANTES in human PBMCs (data not shown).

The effects of CTB or CpG 2006, either alone or in the mixed (CTB + CpG 2006) or conjugated (CTB-CpG 2006) form on induction of proliferative response in human PBMCs were examined. CTB alone did not induce any proliferative response in human PBMCs. Treatment of the cells with CpG 2006, in contrast, gave rise to a strong proliferative response (Table I). However, treatment of the cells with mixture of CTB + CpG 2006 (data not shown) or CTB-CpG 2006 did not confer a further increase in proliferative response over that of CpG 2006 (Table I). Taken together, these data indicate that conjugation of CTB to optimal human CpG 2006 can dramatically potentiate the stimulatory effect of optimal human CpG on proinflammatory cytokine and chemokine responses in vitro without affecting cell proliferation to an appreciable extent.

**CpG motif is critical for the enhanced immunostimulatory effects of CTB-CpG**

Next, we sought to ascertain the relative importance of the CpG motif in the immunostimulatory effects of CTB-CpG. To this end, human PBMCs were cultured in the presence of increasing concentrations of control ODN IMTO22 (a control GpC ODN for CpG 2006 lacking the CpG motif), CTB-IMTO22, CpG 2006, or CTB-CpG 2006. Incubation of the cells with ODN IMTO22 induced low levels of MIP-1α, MIP-1β, and IL-6. At the highest concentration (9 μg/ml), CTB-IMTO22 induced slight increases in MIP-1α, MIP-1β, and IL-6 productions over those of IMTO22-treated cells. As expected, CTB-CpG 2006 induced a dramatic increase in MIP-1α, MIP-1β, and IL-6 responses over those of CpG 2006 (Fig. 2, A–C). Neither IMTO22 nor CTB-IMTO22 induced any significant proliferative response in human PBMCs (data not shown). These results imply that CpG motif is a critical component for immunostimulatory effects of CTB-CpG conjugate.

**Linking of CpG with PO backbone to CTB augments its immunostimulatory effect**

Next, we sought to examine whether immunostimulatory effects of CpG with PO backbone (CpG PO) can also be enhanced by conjugation to CTB. To this end, human PBMCs were incubated with increasing concentrations of CpG 2006 PS, CpG 2006 PO, CTB + CpG PS/CpG PO, CTB-CpG PS, or CTB-CpG PO. Incubation of PBMCs with CpG 2006 PS induced low amounts of MIP-1α, MIP-1β, and IL-6, whereas CpG PO did not induce any appreciable levels of these cytokines. Incubation of PBMCs with CTB-CpG PS resulted in robust MIP-1α, MIP-1β, and IL-6 responses that stayed markedly elevated for 72 h. CTB-CpG PO induced strong MIP-1α, MIP-1β, and IL-6 responses with similar kinetic, but did not reach the levels induced by CTB-CpG PS (Fig. 2, D–F). Interestingly, incubation of PBMCs with either CpG PO or CTB-CpG PO did not result in any appreciable levels of proliferative response (data not shown). These data demonstrate that linkage of CpG PO to CTB converts a molecule with little inherent immunostimulatory effect into a potent stimulator of proinflammatory cytokine and chemokine responses.

**Conjugation of optimal mouse CpG to CTB enhances the immunostimulatory effects of mouse CpG in vitro**

We investigated the immunostimulatory effects of optimal mouse CpG linked to CTB in murine splenocytes. Murine splenocytes were cultivated in the presence of increasing concentrations of CTB, CpG 1826, CTB + CpG 1826, or CTB-CpG and supernatants were collected for cytokine/chemokine analysis and cells assessed for proliferation after 72 h. CTB did not induce any MIP-1α, MIP-1β, and IL-6 productions over those of IMTO22-treated cells. As expected, CTB-CpG induced a significantly stronger splenocyte proliferative response over those of CpG. CTB-CpG, in contrast, induced high levels of proliferative response in a dose-dependent manner. CTB + CpG did not give rise to any additional increase in proliferative response over those of CpG. CTB-CpG, in contrast, induced a significantly stronger splenocyte proliferative response compared with those of CpG (Fig. 3D).

To exclude the possibility that chemical modifications of CTB (maleimide activation) and CpG ODN (thiolation) are responsible for the enhanced immunostimulatory effects of CTB-CpG, murine splenocytes were cultivated with CTB, maleimide CTB, CpG ODN or thiolated CpG ODN. As illustrated in Fig. 3, E and F, the chemical modifications of CTB and CpG ODN did not give rise to enhanced MIP-1α and proliferative responses over those of CTB and CpG, respectively. Thus, the chemical modifications of CTB and CpG ODN are not responsible for the enhanced immunostimulatory effects of CTB-CpG.

To examine the importance of CpG motif in the CTB-CpG-induced immune response, we tested the immunostimulatory effects of a GpC containing ODN (GpC ODN) linked to CTB in

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Table I. The effect of CpG and CTB-CpG on induction of proinflammatory cytokine and chemokines as well as proliferative responses in human PBMCs

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<tr>
<td>IL-1β</td>
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<td>0.8 ± 0.04</td>
<td>134 ± 0.5</td>
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<tr>
<td>IL-8</td>
<td>218 ± 6.2</td>
<td>205 ± 38</td>
<td>993 ± 12</td>
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<tr>
<td>MCP-1</td>
<td>217 ± 1.2</td>
<td>530 ± 25</td>
<td>1951 ± 1</td>
</tr>
<tr>
<td>Proliferative response</td>
<td>112 ± 11</td>
<td>7820 ± 378</td>
<td>5545 ± 117</td>
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*Human PBMCs were incubated with CpG 2006 (3 μg/ml) or CTB-CpG 2006 (CpG content: 3 μg/ml). The cell supernatants were analyzed for IL-1β, IL-8, and MCP-1 (24-h supernatants). Cell proliferative responses were examined after 72 h as described in Materials and Methods. Data shown are representative of two independent experiments and are expressed as nanograms per milliliter per million cells (for IL-1β, IL-8, and MCP-1) and cpm (for proliferative response).
murine splenocytes. Murine splenocytes were cultured in the presence of GpC ODN, CTB-GpC, CpG 1826, or CTB-CpG 1826. GpC ODN induced virtually no MIP-1α, MIP-1β, and proliferative responses in murine splenocytes. CTB-GpC induced a marginal increase in the chemokine response over those of GpC ODN (Fig. 4, A and B). CTB-GpC elicited a more potent proliferative response compared with that of GpC ODN; however, the level of CTB-GpC-induced proliferative response did not reach that of CTB-CpG (Fig. 4C). Taken together, these results indicate that the immunostimulatory effect of optimal mouse CpG can be enhanced by conjugation to CTB and that such conjugation can dramatically lower the concentration of CpG needed for stimulating immune responses in murine splenocytes.

Incubation with GM1 ganglioside diminishes the immunostimulatory effects of CTB-CpG

Given the importance of the GM1 receptor for the cellular uptake of CTB, we examined whether preincubation of CTB-CpG with GM1 ganglioside could influence the immunostimulatory effects of CTB-CpG. To this end, CpG 1826 and CTB-CpG 1826 were preincubated at 37°C for 30 min in the presence or absence of GM1 ganglioside. Murine splenocytes were cultured with CpG 1826, CTB-CpG 1826, GM1-treated CpG 1826, or GM1-treated CTB-CpG 1826 followed by analysis of proliferative and cytokine/chemokine responses. As depicted in Fig. 5, splenocytes incubated with CpG or GM1-treated CpG produced similar levels of MIP-1α. Likewise, comparable levels of proliferative response were observed in splenocytes incubated with CpG or GM1-treated CpG. However, pretreatment of CTB-CpG with GM1 significantly reduced the magnitude of the observed immune responses (Fig. 5 and data not shown). These results suggest that the GM1 receptor-binding property of CTB-CpG is required for its enhanced immunostimulatory effects.

Blocking of endosomal acidification does not abolish CTB-CpG-induced immune response

Previous studies have shown that endosomal acidification is a critical step for the immunostimulatory effects of CpG DNA. To determine whether the CTB-CpG-induced immune response is also

![Graphs showing immune response](http://www.jimmunol.org/DownloadedFrom)
dependent on endosomal acidification, murine splenocytes were pretreated with chloroquine or quinacrine, known as blockers of endosomal acidification (18, 19), followed by an overnight incubation with CpG ODN or CTB-CpG. As depicted in Fig. 6A, chloroquine and quinacrine reduced the CpG-induced MIP-1α production by 11- and 21-fold, respectively. CTB-CpG-induced MIP-1α production, in contrast, was not reduced by chloroquine or quinacrine. Chloroquine and quinacrine reduced the CpG-induced MIP-1α production by 7- and 11-fold, respectively. However, these inhibitors induced only a marginal reduction in CTB-CpG-induced MIP-1α response (data not shown). Similar to CpG, MIP-1α and MIP-1β productions induced by CTB + CpG were dramatically reduced by pretreatment of the cells with chloroquine or quinacrine (data not shown). Consistent with the chemokine data, the CpG ODN-induced proliferative response was markedly reduced by chloroquine and quinacrine (18-fold reduction), whereas these inhibitors did not affect the CTB-CpG-induced proliferative response (data not shown). Together, these results indicate that blocking of endosomal acidification, which dramatically inhibits the CpG-induced immune response, does not abolish CTB-CpG-induced immune responses.

PI3K is involved in CTB-CpG-induced immune activation

PI3K is an important mediator of TLR9 signaling by CpG (20). To examine the involvement of PI3K in CTB-CpG-induced immune responses, mouse splenocytes were incubated with wortmannin (WMN), a PI3K inhibitor (20), for 2 h followed by overnight incubation of the cells with CpG or CTB-CpG. As shown in Fig. 6B, WMN completely blocked the CpG-induced MIP-1α production. WMN reduced the CTB-CpG-induced MIP-1α by 2-fold. Similar pattern was observed in MIP-1β production where CTB-CpG reduced MIP-1β production by 3-fold when cells had been pretreated with WMN (data not shown). Thus, it appears that PI3K activity is involved in CTB-CpG-induced immunity.

Inhibitory ODN 1502 singly or in conjugation with CTB fails to inhibit the CTB-CpG-induced chemokine response

Inhibitory ODNs that contain poly G or GC sequences have been shown to specifically inhibit CpG-induced immunity. Therefore, we wanted to evaluate the effect of inhibitory ODN 1502, with known inhibitory effect on CpG (21) on induction of immunity by
CTB-CpG. Murine splenocytes were cultivated with CpG or CTB-CpG in the presence or absence of increasing concentrations of inhibitory ODN. Inhibitory ODN dose-dependently suppressed the CpG-induced MIP-1α production. Thus, a concentration of 9 μg/ml inhibitory ODN completely blocked MIP-1α production (Fig. 6C) and induced a 5-fold reduction in MIP-β response (data not shown). CTB-CpG-induced MIP-1α (Fig. 6D) and MIP-1β responses (data not shown), in contrast, were not affected by the inhibitory ODN at any concentration used.

Next, we addressed the question of whether inhibitory ODN linked to CTB (CTB-inhib) can inhibit the immunostimulatory effects of CTB-CpG. To this end, murine splenocytes were incubated with increasing concentrations of CTB-inhib conjugate. As depicted in Fig. 6E, CTB-inhib conjugate did not significantly inhibit the CTB-CpG induced MIP-1α response; however, a slight reduction in MIP-1α response was observed when a concentration of 9 μg/ml CTB-inhib was used. Taken together, these results imply that the inhibitory ODN singly or in conjugation with CTB fails to inhibit the CTB-CpG-induced chemokine response.

CTB-CpG-induced immune response is dependent on TLR9 and MyD88

To investigate whether TLR9 and MyD88 are involved in CTB-CpG signaling, splenocytes from C57BL/6, TLR9+/−, and MyD88+/− mice were cultivated in the presence of CpG ODN or CTB-CpG. Splenocytes from C57BL/6 mice produced a significant amount of MIP-1α in response to CpG ODN, which was further enhanced by 5-fold in response to CTB-CpG (data not shown). As expected, splenocytes from TLR9−/− and MyD88−/− mice had no MIP-1α response to CpG ODN. Similarly, CTB-CpG did not induce any MIP-1α response in splenocytes from TLR9−/− or MyD88−/− mice. However, splenocytes from TLR9−/− mice produced significant amounts of MIP-1α in response to LPS while no detectable level of MIP-1α was produced by splenocytes from MyD88−/− mice cultivated with LPS (Fig. 7A). Similar to C57BL/6 mice, splenocytes from TLR9+/− heterozygote mice produced elevated levels of MIP-1α in response to CpG and CTB-CpG (data not shown). Neither CpG ODN nor CTB-CpG induced any appreciable levels of proliferative responses in splenocytes from TLR9−/− and MYD88−/− mice. However, LPS induced a similar significant level of proliferative response in splenocytes from C57BL/6 and TLR9−/− mice (Fig. 7B). These data indicate that similar to CpG, the CTB-CpG-induced immune response is critically dependent on TLR9 and MyD88.

Enhanced immune response elicited by CTB-CpG is not attributed to LPS and is not mediated by TLR4

We evaluated the possible involvement of LPS and also TLR4 in CTB-CpG-induced immune responses by using C3H/HeJ (TLR4-deficient) and C3H/HeN (wild-type) mice. Thus, splenocytes from C3H/HeN and C3H/HeJ mice were cultivated in the presence of CpG or CTB-CpG. CpG induced similar levels of MIP-1α, MIP-1β, and proliferative responses in splenocytes from C3H/HeN and C3H/HeJ mice (Fig. 7, A and B, and data not shown). Likewise, comparable levels of MIP-1α, MIP-1β, and proliferative responses were mounted by splenocytes from C3H/HeN and C3H/HeJ mice in response to CTB-CpG. As expected, splenocytes from C3H/HeN, but not C3H/HeJ, mice proliferated and produced significant amounts of MIP-1α and MIP-1β in response to LPS (Fig. 7, A and B, and data not shown). We further investigated the possible involvement of LPS in CTB-CpG-induced immune responses by incubation of splenocytes from C57BL/6 mice with CpG, CTB-CpG, or CTB-CpG in the presence or absence of polymyxin B. As shown in Fig. 7C, comparable levels of MIP-1α were produced by the splenocytes in the presence or absence of polymyxin B. These data clearly demonstrate that the enhanced immunostimulatory

![FIGURE 4](https://www.jimmunol.org/)

CpG sequence is important for the enhanced immunostimulatory effects of CTB-CpG in mouse splenocytes. Splenocytes from C57BL/6 mice were incubated with CpG 1826, CTB-CpG 1826, CpG ODN, or CTB-CpG. The supernatants were analyzed after 24 h for MIP-1α. Cell proliferative responses were examined on day 3 (A). Results shown are representative of two independent experiments and expressed as mean of duplicate ELISA samples or triplicate proliferation samples ± SEM.

![FIGURE 5](https://www.jimmunol.org/)

Incubation with GM1 ganglioside diminishes the immunostimulatory effects of CTB-CpG. A and B, CpG 1826 (0.3 μg/ml) or CTB-CpG 1826 (CpG content: 0.3 μg/ml) were preincubated at 37°C for 30 min in the presence or absence of GM1 ganglioside (2.5 or 10 μg/ml). Splenocytes from C57BL/6 mice were incubated with the preincubated reagents. MIP-1α was measured in the supernatants by ELISA after overnight incubation (A). Cell proliferative responses were examined on day 3 (B). Results shown are representative of two different experiments and are presented as the mean of duplicate ELISA samples or triplicate proliferation samples ± SEM. * and **, Statistically significant by Student’s t test at p < 0.05 and p < 0.01, respectively, compared with groups without GM1.
property of CTB-CpG is not mediated by LPS and that TLR4 is not involved in CTB-CpG-induced immune responses.

CTB-CpG-induced immune response is dependent on NF-κB

Many genes involved in immunostimulatory responses are activated by the transcription factor NF-κB. To investigate whether CTB-CpG activation of TLR9-MyD88 signaling was mediated through NF-κB, human PBMCs were exposed to CTB, CpG 2006, CTB + CpG 2006, or CTB-CpG 2006 for up to 2 h. Nuclear extracts were prepared and subjected to EMSA and supershift analysis for determination of NF-κB DNA-binding activity. Nuclear extracts from nonstimulated cells were used as controls. As illustrated in Fig. 8, NF-κB was activated by CTB-CpG conjugate and to a lesser extent by CpG ODN, with p65 identified as the major activated NF-κB subunit. Minimal activation of NF-κB above that observed with control cells occurred when CTB and CTB + CpG ODN were tested. To further confirm the involvement of NF-κB, the impact of gliotoxin, a specific inhibitor of NF-κB on the CTB-CpG-induced immune responses were investigated. Mouse splenocytes were preincubated in the presence or absence of gliotoxin for 2 h. The preincubated cells were then exposed to CpG OD1 1826 or CTB-CpG 1826 for 4 and 24 h. The treatment of the cells with gliotoxin abolished the MIP-1α and proliferative responses produced by CpG and CTB-CpG at both time points (data not shown). Hence, similar to CpG, the CTB-CpG-induced immune response is mediated by NF-κB.

Conjugation of CpG to CTB confers an enhanced cross-species activity to CpG

Different CpG motifs are optimal to activate murine and human immune cells. Thus, PO ODNs containing the optimal murine CpG motif, used at concentrations that are highly active on murine immune cells, showed little or no immunostimulatory activity on human immune cells (22). We were interested to know whether conjugation of CTB to CpG can override the species specificity effect of CpG. Hence, human PBMCs were cultivated in the presence of increasing concentrations of optimal mouse CpG 1826, CTB + CpG 1826, or CTB conjugated to CpG 1826. Incubation of human PBMCs with optimal mouse CpG 1826 at a concentration of 27 μg/ml induced a 2-fold increase in MIP-1α production, which was
not further enhanced when CTB + CpG was used. CTB-CpG 1826, in contrast, induced a robust MIP-1α response in a dose-dependent manner. Thus, a concentration of 27 μg/ml CTB-CpG induced a 418-fold increase in MIP-1α production over untreated PBMCs (Fig. 9A). CpG (27 μg/ml) induced a 19-fold increase in MIP-1β response compared with untreated PBMCs that was not further increased when CTB + CpG was used. Conversely, incubation of human PBMCs with CTB-CpG resulted in a 205-fold increase in MIP-1β level as compared with untreated PBMCs (Fig. 9B). Thus, conjugation of optimal murine CpG to CTB confers a strong immunostimulatory effect on human PBMCs. We then tested the immunostimulatory effects of human CpG 2006 linked to CTB in murine splenocytes. Splenocytes from C57BL/6 mice produced moderate levels of MIP-1α (4000 pg/ml/million cells) and MIP-1β (10 000 pg/ml/million cells) in response to CpG 2006 (3 μg/ml) that was not further enhanced when CTB + CpG 2006 was used. CTB-CpG 2006 induced a 3-fold increase in MIP-1α and MIP-1β levels over those of CpG 2006 (Fig. 9, C and D). We also tested the requirement of CpG sequence in the cross-species activity of CTB-CpG in murine splenocytes. Incubation of splenocytes from C57BL/6 mice with non-CpG-containing control ODN IMTO22 induced virtually no MIP-1α, MIP-1β, and IL-6 responses. However, CTB-IMTO22 induced a slight increase in cytokine/chemokine responses (data not shown).

Next, we examined whether the cross-species activity of CpG ODN was mediated by TLR9 and MyD88. Thus, splenocytes from TLR9+/− and MyD88−/− mice were cultivated with human CpG 2006, CTB + CpG 2006, or CTB-CpG 2006. As depicted in Fig. 9, C and D, incubation of splenocytes from TLR9+/− and MyD88−/− mice with CpG 2006, CTB + CpG 2006, or CTB-CpG 2006 did not result in any significant MIP-1α and MIP-1β responses. Together, these results indicate that conjugation of CpG ODN to CTB confers an enhanced cross-species activity to CpG ODN and that the cross-species activity of CTB-CpG is also mediated by TLR9 and MyD88.

**Conjugation of CTB to CpG enhances adjuvanticity of CpG**

Given the potent immunostimulatory effects of CTB-CpG, we sought to compare the adjuvanticity of CTB-CpG with CpG, CTB, and CTB + CpG. Groups of C57BL/6 mice were s.c. injected with 10 μg of TT alone, or together with either 5 μg of CpG ODN, 18 μg of CTB, or equivalent amounts of CpG or CTB in the mixture or conjugate, two times, with a 1-wk interval. Three weeks after the last immunization, the level of serum IgG Ab against TT was examined. The TT vaccinated group had considerable amounts of TT-specific IgG in their sera. Immunization with TT together with CTB, CpG ODN, or CTB + CpG induced almost 2-fold increase in TT-specific IgG Ab responses compared with TT immunization alone. However, mice immunized with TT together with CTB-CpG induced a 6-fold increase in their TT-specific IgG Abs as compared with TT group (Fig. 10A). These data imply that CTB-CpG is a strong adjuvant for induction of systemic Ab responses against a coadministered Ag.

Next, we tested the levels of IgG1 and IgG2c Abs as indicator of Th2 and Th1 type responses in C57BL/6 mice, respectively, in the sera of immunized mice. As depicted in Fig. 10B, all vaccinated groups had comparable levels of a specific IgG1 Ab response. By contrast, the level of IgG2c Ab varied. Thus, mice immunized with TT, or TT coadministered with CTB or CTB + CpG, had low levels of serum IgG2c. Mice immunized with TT together with CpG had moderate levels of IgG2c (a 2-fold increase over TT alone), whereas immunization with TT coadministered with CTB-CpG induced a dramatic increase in the TT-specific IgG2c levels (a 6-fold increase over TT alone), indicating of a strong Th1-tilting effect of CTB-CpG on immune response in vivo (Fig. 10C).

We then examined IFN-γ response, a bona fide marker of cellular immune response, in splenocytes isolated from the immunized mice upon Ag recall stimulation in vitro. Splenocytes from
the immunized mice were cultured with TT for 48 h and IFN-γ production was analyzed in cell culture supernatants. Although splenocytes from mice immunized with TT alone or TT together with CTB, CpG ODN, or CTB/CpG did not produce any appreciable levels of IFN-γ, splenocytes from mice immunized with TT together with CTB-CpG produced considerable amounts of IFN-γ in vitro (Fig. 10D).

Discussion

In this study, we describe the development and properties of a potent novel immunostimulatory agent based on chemical conjugation of CpG ODN to CTB. The rationale for this construction involved the possibility of combining the immunostimulatory properties of CpG ODN with the efficient ability of CTB to bind to immune cells, especially APCs, and to transport linked agent into endosomes and retrogradely further to the TGN and the ER. Optimal human CpG 2006 linked to CTB was shown to mount robust proinflammatory cytokine and chemokine responses in human PBMCs while CpG 2006 alone or an admixture of CTB and CpG induced only weak responses. Also in mouse splenocytes, optimal murine CpG linked to CTB mounted a more potent proinflammatory cytokine and chemokine responses as well as proliferative response than CpG alone or admixture of CTB and CpG. Interestingly, the well-documented species specificity of CpG ODN appears to be largely overridden by the binding to CTB. Of special significance, CTB-CpG displayed a markedly augmented in vivo adjuvant activity for a coadministered vaccine Ag, TT compared with CpG alone, or a mixture of CTB and CpG. We also report a series of studies that analyze mode of action of CTB-CpG. In the following, we attempt to discuss several possibilities that may depict the underlying mechanism for the enhanced immunostimulatory properties of CTB-CpG.
We could show that the immunostimulatory properties of CTB-CpG are dependent on the presence of CpG motif as a non-CpG-containing ODN conjugated to CTB failed to elicit robust proinflammatory cytokine and chemokine responses in human PBMCs. Furthermore, and despite dissimilarities between CpG and CTB-CpG discussed below, several lines of evidence in the present study suggest that CTB-CpG shares critical molecules involved in TLR9 signaling with CpG. Thus, similar to CpG, the CTB-CpG-induced immune response was found to be dependent on TLR9, MyD88, PI3K, and NF-κB.

In our mouse in vitro experiments, we could show that the CpG dose required for eliciting proinflammatory cytokine and chemokine responses as well as proliferative response can be dramatically reduced if CpG is linked to CTB. Furthermore, preincubation of CTB-CpG with gangioside GM1 was shown to decrease the response produced by CTB-CpG to that of CpG ODN. It is therefore likely that, in addition to the endosomal formation evidenced for CpG uptake, the cellular uptake of CTB-CpG through binding to the GM1 receptor allows more CpG to reach the endosomal compartments. Of note, the recent report of Yasuda et al. (23) revealed that enhancement of endosomal translocation of CpG DNA by using the cationic lipid DOTAP augments the immunostimulatory effects of CpG DNA in dendritic cells in terms of up-regulation of CD40/CD69 and production of type I IFN and IL-6.

Recent studies of Leifer et al. (24) demonstrated that TLR9 is localized in the ER before CpG exposure. Concurrent with the movement of CpG in target cells, TLR9 redistributes from the ER to the endosome (25). Internalized CTB has been found to traffic intracelluarity via a retrograde vesicular pathway to reach the TGN, Golgi apparatus, and eventually the ER (13). One possibility is that CTB-CpG might follow the route used by CTB and thereby translocates to the ER where CpG can primarily interact with TLR9. CpG-TLR9 may then translocate from the ER to the endosomal vesicles where the TLR9 signaling takes place. It has been recently reported that retention of CpG in the endosomal vesicle may enhance the avidity of CpG to TLR9. Thus, monocyte-derived human DCs that respond poorly to CpG-A mount a robust IFN induction if CpG-A is manipulated using a cationic lipid for endosomal retention (26). It is therefore possible that conjugated CTB-CpG is retained for longer periods in the endosomal vesicle thus enhancing the avidity of CpG to TLR9. Cell imaging studies are underway to investigate this possibility. Additionally, the resistance of the immune responses produced by CTB-CpG to the inhibitory ODN suggests that unlike CpG, the interaction of CTB-CpG with TLR9 does not primarily take place in endosomal vesicles and further stress our notion that the ER might be involved in this process. Nonetheless, the inhibitory ODN linked to CTB failed to diminish the CTB-CpG-induced immune response. This illustrates the complexity of the underlying mechanism of CTB-CpG.

Recent observations by Wu et al. (27) indicate that multimerization of CpG ODN plays a pivotal role for TLR9 activation and that CpG PO can also activate TLR9 if they form a multimeric structure. Our findings show that CpG PO linked to CTB can mount a potent immune response. By analogy, it is therefore possible that coupling of CTB, which has a pentameric structure, to CpG effectively, confers CpG aggregation/multimerization that may subsequently lead to a more effective interaction of CpG with TLR9. Consistent with this notion, it has been recently shown that adsorption of CpG ODN to the surface of cationic poly (DL-lactide-co-glycolide) microspheres enhances the secretion of IFN-γ and IFN-α from human PBMC compared with CpG ODN alone (8). Several studies have also shown that CpG ODN linked to protein Ags elicit a more potent Ag-specific response than admixture of CpG ODN and Ag (28–30).

Another interesting feature of CTB-CpG explained in our study is its potent adjuvant activity for induction of an Ag-specific IgG Ab response when used for s.c. immunization together with a model vaccine Ag, TT, in C57BL/6 mice. We found that CTB-CpG is especially capable of mobilizing more potent IgG2c Ab and IFN-γ responses than CpG, indicating that coupling of CpG to CTB tilts the resulting immune response toward Th1 type immunity. Previous studies point to a synergistic adjuvant activity between CpG ODN and CT or LT for induction of Ag-specific immune responses in mice. In one study, transcutaneous immunization of mice with Chlamydia trachomatis major outer membrane protein as Ag and a mixture of CpG and CT as adjuvant was shown to be superior to CpG alone in inducing immune responses against the coadministered Ag (10). However, the inherent toxicity of CT and LT precludes their use as adjuvants in humans. Consistent with our findings, a mixture of CpG ODN and nontoxic derivatives of CT and LT, i.e., CTB and the mutant of LT protein derivative LTκ63, had no synergistic effects on the magnitude of the Ag-specific immune response (9). Conjugation of CpG and CTB may be a means of overcoming these functional limitations.

In conclusion, we described here the development and properties of a potent immunostimulatory agent based on chemical conjugation of CpG ODN to CTB. We found that the immunostimulatory and the adjuvant effects of CpG can be dramatically enhanced by the conjugation to CTB. To our knowledge, we are the first to report CTB as the first nontoxic derivative of microbial toxins discovered that when chemically linked to CpG ODN dramatically enhances the CpG ODN-mediated immune responses. This observation warrants further exploration of the impact of the new class of immunostimulatior/adjuvant based on CpG ODN linked to CTB for inducing more potent immune responses.

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Disclosures

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