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Involvement of antigen-presenting cells in the enhancement of the *in vitro* antibody responses by cholera toxin B subunit

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SUMMARY

The enhancing effect of cholera toxin B subunit (CTB) on primary antibody responses to keyhole limpet haemocyanin (KLH) and the cellular basis of the effect were investigated, using *in vitro* cultures of mouse spleen cells. CTB (1–100 ng/ml) enhanced anti-KLH IgM, IgG and IgA antibody responses in a dose-dependent manner, when added to the cultures with KLH. This immunoenhancement was antigen specific, but not due to either polyclonal activation of the spleen cells or antigenic cross-reactivity between CTB and KLH. CTB did not affect the kinetics of the anti-KLH antibody responses. Early (Days 0–1) addition of CTB to the cultures enhanced the anti-KLH antibody production, whereas late (Days 5–7) addition of CTB did not. Addition of CTB with KLH to splenic adherent cells (SAC) resulted in a dose-dependent enhancement of the anti-KLH antibody responses, when the SAC were reconstituted with unimmunized non-adherent cells. Moreover, CTB enhanced IL-1 secretion from SAC incubated with KLH. These results suggest that CTB enhances the primary anti-KLH antibody responses *in vitro* by acting on early events in the responses, and that antigen-presenting cells play a major role in the enhancement.

INTRODUCTION

Cholera toxin (CT), a bacterial exotoxin derived from *Vibrio cholerae*, is composed of an A subunit which elevates intracellular levels of cyclic adenosine monophosphate (cAMP) by activation of adenylate cyclase, and five B subunits which bind to cell membrane GM_1 ganglioside with high affinity.[1–3] Although CT is widely known to be a potent adjuvant on immune responses *in vivo*,[4–8] we demonstrated that the B subunit of CT (CTB) also markedly enhanced antibody responses to unrelated protein antigens which were administered together with CTB into mice.[9,10] Other investigators reported that antigens conjugated to CTB induced enhanced antibody responses and, therefore, suggested that CTB functioned as a carrier protein.[11,12] However, the mechanism by which CTB enhances antibody responses to CTB-unconjugated proteins so far remains unclear.

Recently, it has been demonstrated that CTB has various effects on murine lymphocytes *in vitro*: CTB inhibited both mitogen- or antigen-induced T-cell proliferation and anti-IgM-induced B-cell proliferation.[13] CTB also promoted isotype differentiation of lipopolysaccharide-stimulated B cells.[14] Moreover, CTB was mitogenic for rat thymocytes.[15] These findings suggest that the mechanism of immunoenhancement by CTB may involve direct effects of CTB on the cells of the immune system.

In this study, we examined the cellular basis of the immunoenhancing effect of CTB, using an *in vitro* antibody production system with mouse spleen cells. The data indicate enhancement by CTB of *in vitro* primary antibody responses to keyhole limpet haemocyanin (KLH), and further, substantial involvement of antigen-presenting cells (APC) in the enhancement. On the basis of these results, the possible mechanisms of the *in vivo* adjuvant action of CTB are discussed.

Abbreviations: APC, antigen-presenting cells; cAMP, cyclic adenosine monophosphate; CT, cholera toxin; CTB, cholera toxin B subunit; ELISA, enzyme-linked immunosorbent assay; IL-1, interleukin-1; KLH, keyhole limpet haemocyanin; SAC, splenic adherent cells.

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MATERIALS AND METHODS

Animals

Female C57BL/10 mice were obtained from Japan SLC Inc., Hamamatsu, Japan and used at 22–26 weeks of age.

Antigen and agent

KLH was purchased from Calbiochem Corp., La Jolla, CA. CTB was obtained from Sigma Chemical Co., St Louis, MO. The purity of the CTB preparation used in these studies was verified by intracellular cAMP assay with CTB-treated CHO cells, sodium dodecyl sulphate-polyacrylamide gel electrophor-

esis and the Limulus and rabbit pyrogen tests, as described previously.[10]

Whole-spleen cell culture

Spleens were removed from intact mice and teased to create a single-cell suspension. After lysis of erythrocytes, cells were washed in RPMI-1640 media (Nissui Pharmaceutical Co., Tokyo, Japan), and resuspended at 10^7 cells/ml in RPMI-1640 supplemented with 10% foetal calf serum (Gibco, Chagrin Falls, OH), 2 mM L-glutamine (Flow Lab., McLean, VA), 50 μM 2-mercaptoethanol (Wako Junyaku Co., Osaka, Japan), 5 mM HEPES (Gibco), 1·6 mg/ml sodium bicarbonate and 60 μg/ml kanamycin (Meiji Seika Co., Tokyo, Japan). The cells were incubated with KLH and/or CTB in 24-well plates (Linbro, Flow Lab.) at 37 and 5% CO_2. After 24 hr of culture, the cells were washed, resuspended in fresh medium, and then cultured for an additional 6 or 10 days. Culture fluids were replaced by fresh media every 48 hr, and the fluids were centrifuged and stored at −40 for later antibody titration. In other experiments CTB was added to cultures for periods other than the first 24 hr.

Antibody assay

The levels of anti-KLH IgM, IgG and IgA antibodies in the samples were measured by enzyme-linked immunosorbent assay (ELISA), as described elsewhere.[9,16] Briefly, KLH-coated wells of an ELISA plate (Costar, Cambridge, MA) were sequentially incubated with the samples, alkaline phosphatase-coupled goat anti-mouse IgM, IgG or IgA (μ-, γ- or α-chain specific; Zymed Lab., San Francisco, CA), and finally *p*-nitrophenyl phosphate (Sigma). The chromogen produced was measured by absorbance at 405 nm in a SJ Auto Reader (Sanko Junyaku Co., Tokyo, Japan). As a 16-unit standard for each antibody, a constant dilution (approximately $1:10^3$ to $1:10^6$) of pooled serum from mice hyperimmunized with KLH was used. The antibody concentration of unknown samples was expressed in units of standard.

The concentration of total IgM or IgG was also determined by ELISA. The procedure was the same as described above, except that the plate was coated with goat anti-mouse IgM or IgG (Zymed), and that purified mouse IgM or IgG (Cappel Products, West Chester, PA) was used as a standard for 1000 ng/ml of total IgM or IgG.

Cell separation and reconstitution

Splenic adherent cells (SAC) were prepared by the method described previously[17] with minor modifications. In brief, whole-spleen cells were incubated in foetal calf serum-precoated plastic dishes (Falcon, Becton Dickinson Co., Lincoln Park, NJ) at 37 for 60 min. The dishes were repeatedly rinsed to remove completely non-adherent cells. Adherent cells were then treated with anti-Thy-1.2 monoclonal antibody (Meiji Nyugyo Co., Tokyo, Japan) and guinea-pig complement (Denka Seiken Co., Tokyo, Japan). The resulting cell population contained no detectable Thy-1.2$^+$ cells and less than 2% of surface-Ig$^+$ cells as determined by immunofluorescence staining. Splenic non-adherent cells were prepared as described elsewhere.[18] Briefly, spleen cells were incubated in serum-uncoated dishes at 37 for 2 h, and non-adherent cells containing T cells and B cells were collected.

SAC were cultured with KLH alone or with KLH plus CTB for 24 hr and then washed. The immunized SAC at 8×10^4 cells/ml were reconstituted with 8×10^6 cells of unimmunized splenic non-adherent cells, and cultured for an additional 6 days. Culture fluids were replaced and stored as described above.

IL-1 production and assay

SAC at 4×10^6 cells/ml were incubated with KLH in the absence or presence of CTB in culture medium containing 1% foetal calf serum. After 24 hr of culture, the supernatants were collected for interleukin-1 (IL-1) assay.

IL-1 activity was determined by the thymocyte co-mitogenic assay.[19] Briefly, thymocytes from C3H/He mice (Japan SLC Inc.) were cultured with various dilutions of the supernatants in the presence of phytohemagglutinin-P (Difco, Detroit, MI) for 72 hr. Before addition to the culture, all supernatants were incubated with 10 nmol/ml of GM_1 ganglioside (Bachem Inc., Torrance, CA) to protect the thymocytes from any direct effect of CTB remaining in the supernatants; the GM_1 itself was shown to have no effect on cell proliferation. The proliferation of thymocytes was estimated by the reduction of a tetrazolium salt, MTT.[20] In brief, culture fluids of the thymocytes were replaced with fresh media containing MTT (Sigma) and the thymocytes were recultured for 4 hr. Formazan crystals generated by thymocytes were dissolved in isopropanol and measured by absorbance at 570 nm in a SJ Auto Reader. Thus, the IL-1 activity was expressed as the optical density.

Statistical analysis

Results were expressed as the geometric means $\pm$ 1 standard deviation of the original distribution. The significance of differences between experimental groups was analysed by the unpaired form of Student's *t*-test. Probability (*P*) values $< 0{\cdot}05$ were considered significant.

RESULTS

Dose-dependent effect of CTB on anti-KLH antibody responses

Whole-spleen cells, prepared from intact mice, were incubated with 2 μg/ml of KLH and various doses of CTB for the first 24 hr of culture. Culture fluids were replaced every 48 hr, and the fluids on Day 7 were assayed for anti-KLH IgM, IgG and IgA antibody titres. As shown in Fig. 1, addition of 1–100 ng/ml of CTB to the cultures caused a dose-dependent enhancement of the anti-KLH antibody production. However, higher doses of CTB (1–10 μg/ml) depressed the antibody production to background levels. Among IgM, IgG and IgA anti-KLH antibody responses, the same dose–response patterns were observed. These results were reproducible when different doses of KLH were added to the cultures and when a different lot of CTB was used (data not shown).

Antigen specificity of the CTB effect

In order to investigate whether the immunoenhancing effect of CTB described above is antigen specific or polyclonal, the ratio of anti-KLH IgG level to total IgG concentration and that of anti-KLH IgG level to viable cell number were determined for each culture. Both ratios increased with increasing CTB dose (Fig. 2). These ratios for IgM also increased in a dose-dependent fashion (data not shown). Furthermore, cultures which had been incubated with CTB alone produced no detectable levels of

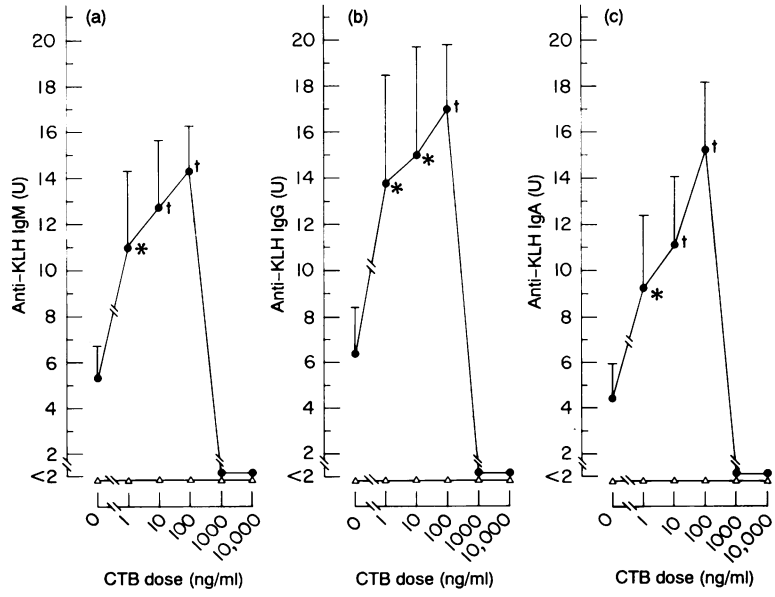

Figure 1. Effect of CTB on anti-KLH antibody responses. Mouse spleen cells were incubated with 2 μg/ml KLH and various doses of CTB (●) or with CTB alone (△) for the first 24 hr of culture. Culture media were replaced every 48 hr, and anti-KLH IgM (a), IgG (b) and IgA (c) antibody titres in culture supernatants were measured by ELISA on Day 7. Each point represents the mean + SD of the titres from quadruplicate cultures. *$P < 0.05$ or †$P < 0.01$ for differences from the group incubated with KLH alone.

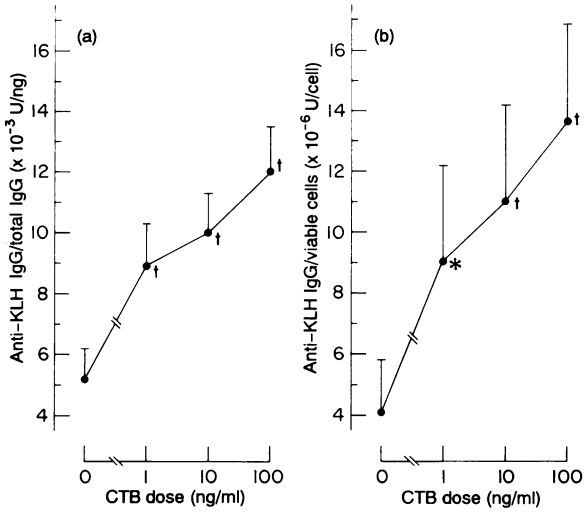

Figure 2. Specificity of the enhancement by CTB of anti-KLH antibody responses. Cells were incubated with 2 μg/ml KLH and various doses of CTB for the first 24 hr. Anti-KLH IgG antibody titres and total IgG concentrations in culture supernatants and viable cell numbers were determined on Day 7, and the ratios of anti-KLH IgG to total IgG (a) and those of anti-KLH IgG to cell numbers (b) were calculated. For other details, see legend to Fig. 1.

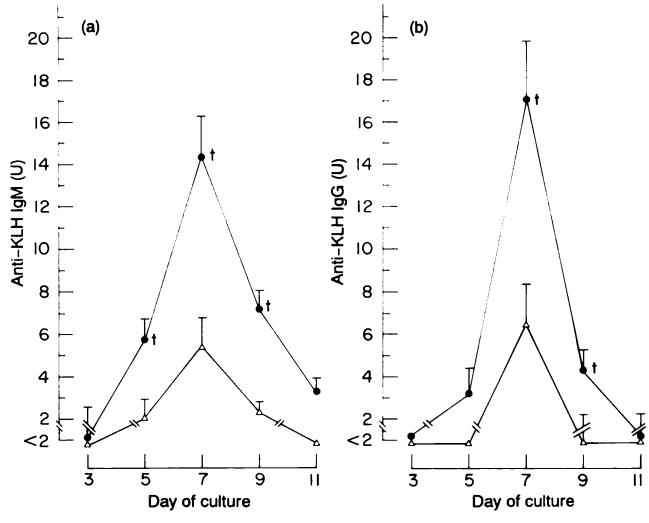

Figure 3. Effect of CTB on the kinetics of anti-KLH antibody responses. Cells were incubated with 2 μg/ml KLH with (●) or without (△) 100 ng/ml CTB for the first 24 hr. Anti-KLH IgM (a) and IgG (b) antibody titres in culture supernatants were measured on Days 3, 5, 7, 9 and 11. For other details, see legend to Fig. 1.

anti-KLH IgM, IgG and IgA antibodies on Day 7, regardless of CTB dose (open triangles in Fig. 1). Thus, relatively low concentrations of CTB (1–100 ng/ml) induce antigen-specific immunoenhancement.

It was also examined whether higher concentrations of CTB cause antigen-specific immunosuppression. Total IgG and IgM concentrations, total cell number and per cent cell viability were markedly lower in cultures incubated with high doses of CTB (1–10 μg/ml) plus KLH than in those incubated with low doses of CTB (1–100 ng/ml) plus KLH or with KLH alone, respectively (data not shown). Similar findings were observed between the groups treated with high doses of CTB alone and those treated with low doses of CTB alone (data not shown). Thus, the decrease in antibody production by relatively high doses of CTB (1–10 μg/ml) is not antigen specific.

Kinetics of the immunoenhancement by CTB

The temporal kinetics of the enhancement of anti-KLH antibody responses by CTB was investigated. Spleen cells were

Table 1. Effect of time of CTB treatment on anti-KLH antibody responses*

Treatment				Anti-KLH antibody titres			
KLH		CTB		Day 7		Day 9	
Dose (μg/ml)	Period (days)	Dose (ng/ml)	Period (days)	IgM (U)†	IgG (U)†	IgM (U)†	IgG (U)†
2	0–1	0	—	6·0±1·6	6·2±1·7	2·2±0·8	1·2±0·8
2	0–1	100	0–1	18·1±4·0‡	19·7±3·7‡	7·5±1·8‡	3·9±0·9‡
2	0–1	100	1–3	7·2±2·4	6·0±2·1	2·9±1·0	1·1±0·8
2	0–1	100	5–7	3·6±1·3§	4·6±1·5	1·5±0·7	1·0±0·5

* Spleen cells were incubated with 2 μg/ml KLH for the first 24 hr and with 100 ng/ml CTB for different periods. Anti-KLH IgM and IgG antibody titres in culture supernatants were measured by ELISA on Days 7 and 9.

† Values represent the means±SD of the titres from quadruplicate cultures.

‡ $P<0·01$ for differences from group incubated with KLH alone.

§ $P<0·05$ for differences from group incubated with KLH alone.

Table 2. Effect of CTB treatment of SAC on anti-KLH antibody responses*

	Treatment of SAC		Anti-KLH antibody titres	
Exp.	KLH (μg/ml)	CTB (ng/ml)	IgM (U)†	IgG (U)†
1	0	0	1·2±0·7‡	1·1±0·7‡
	2	0	4·0±1·2	3·8±1·0
	2	10	8·9±2·2‡	8·7±1·8‡
	2	100	10·1±2·2‡	9·9±1·9§
	2	1000	12·1±2·5§	12·6±2·6§
2	2	0	3·4±0·7	3·3±0·8
	2	100	9·3±1·7§	9·0±1·8§
	2	1000	11·3±1·9§	11·7±2·5§
	2	10,000	14·1±2·7§	14·1±3·0§

* SAC were incubated with 2 μg/ml KLH and various doses of CTB for 24 hr, and then co-cultured with unimmunized splenic non-adherent cells. Anti-KLH IgM and IgG antibody titres in culture supernatants were measured by ELISA on Day 7.

† Values represent the means±SD of the titres from triplicate cultures.

‡ $P<0·05$ for differences from group incubated with KLH alone.

§ $P<0·01$ for differences from group incubated with KLH alone.

incubated with 2 μg/ml of KLH with or without 100 ng/ml of CTB for the first 24 hr. Culture fluids were replaced every 48 hr and each fluid was assayed for anti-KLH IgM and IgG antibody levels. As shown in Fig. 3, CTB-treated cultures produced higher levels of these antibodies than did CTB-untreated cultures at each time-point. Peak production of these antibodies was observed in the samples collected on Day 7, whether or not CTB had been added to the cultures. Thus, CTB has no influence on the kinetics of the anti-KLH antibody responses, although CTB enhances the responses themselves.

Influence of time of CTB treatment on the immunoenhancement

It was examined which phase(s) of anti-KLH antibody responses CTB affects to result in enhancement of the responses. Spleen cells cultured with 2 μg/ml of KLH for the first 24 hr were incubated with 100 ng/ml of CTB for different periods, and anti-KLH IgM and IgG antibody titres were measured on Days 7 and 9. The results are summarized in Table 1. Early (Days 0–1) treatment with CTB induced a significant enhancement of the antibody responses as already shown. In contrast, late (Days 5–7) treatment with CTB caused a mild inhibition of the responses. This inhibition was not accompanied with a decrease in viable cell numbers (data not shown). Treatment with CTB for Days 1–3 caused neither enhancement nor inhibition. These findings suggest that CTB enhances the anti-KLH antibody responses by acting on early events involved in induction of the responses.

SAC-mediated immunoenhancement by CTB

Whether CTB has an effect on SAC population to enhance anti-KLH antibody responses was investigated by cell separation and reconstitution. SAC, purified from spleens of intact mice, were incubated with 2 μg/ml of KLH and various doses of CTB for 24 hr, and then reconstituted with unimmunized splenic non-adherent cells. Anti-KLH IgM and IgG antibody levels in culture supernatants were assayed on Day 7. Table 2 presents the data from two experiments. The cultures immunized with KLH plus CTB produced higher levels of the antibodies than did those immunized with KLH alone, in each experiment. Moreover, the antibody levels increased with an increase of CTB dose. Thus, CTB enhances the anti-KLH antibody responses through its effect on SAC.

Enhancement of IL-1 secretion from SAC by CTB

It was determined whether addition of CTB to SAC cultures at the time of incubation with KLH causes enhancement of IL-1 production from the SAC. SAC were cultured with 2 μg/ml of KLH and various doses of CTB for 24 hr, and the culture supernatants were then assayed for IL-1 activity. As summarized in Table 3, IL-1 levels were higher in cultures incubated with KLH and CTB than in those incubated with KLH alone, and furthermore, the level was higher in the group cultured with 10 μg/ml CTB plus KLH than in that cultured with 100 ng/ml CTB

Table 3. Effect of CTB on IL-1 secretion from SAC*

Treatment		IL-1 activity†
KLH (μg/ml)	CTB (ng/ml)	(OD_{570})
2	0	0·094 ± 0·019‡
2	100	0·193 ± 0·051‡
2	10,000	0·230 ± 0·048‡
2	10,000	0·098 ± 0·030§

* SAC were incubated with 2 μg/ml KLH and various doses of CTB for 24 hr, and then IL-1 activity in the culture supernatants was determined by thymocyte co-mitogenic assay. For details, see Materials and Methods.

† Values represent the means ± SD of the activity from triplicate cultures.

‡ Dilutions (1/3) of culture supernatants were assayed.

§ Dilutions (1/10) of culture supernatants were assayed.

plus KLH. Table 3 also shows that 10 μg/ml of CTB induced an approximately threefold increase in IL-1 over CTB-untreated cultures. Thus, CTB enhances IL-1 secretion from SAC when they are incubated with KLH.

DISCUSSION

The present study was designed to investigate the enhancing effect of CTB on the *in vitro* primary antibody responses to KLH, and the cellular basis of the effect. Addition of nanogram amounts of CTB to cultures stimulated with KLH caused a dose-dependent enhancement of the anti-KLH antibody responses together with an increase in the specific activity of the antibodies (Figs 1 and 2). Cultures incubated with CTB in the absence of KLH produced no detectable levels of the anti-KLH antibodies (Fig. 1). These indicate that the immunoenhancement by CTB is attributed to neither polyclonal activation and non-specific proliferation of B cells, nor antigenic cross-reactivity between CTB and KLH. The possibility that the enhanced anti-KLH antibody responses are due to an alteration of the kinetics of the antibody responses by CTB is also eliminated, because CTB did not affect the kinetics (Fig. 3). Taken together, these results show that CTB substantially and specifically enhances the antibody responses to immunogens which are present with CTB in cultures. In contrast, higher doses of CTB markedly depressed the anti-KLH antibody production together with cell viability and total cell number (Fig. 1). This suppression would be at least in part attributable to cytotoxicity of CTB. Since microgram amounts of CTB have been shown to inhibit mitogen- or antigen-induced lymphocyte proliferation *in vitro*,[13,21] such an inhibitory effect may be also involved in the suppression observed here.

Early treatment of KLH-stimulated cultures with CTB enhanced anti-KLH antibody responses regardless of the isotype of the antibody, whereas the late treatment failed to enhance the responses (Table 1). Therefore, we considered that the cells which participate at early stages of the antibody responses were most affected by CTB. In reconstitution studies using SAC and splenic non-adherent cells, addition of CTB to KLH-stimulated SAC resulted in enhancement of the anti-KLH antibody responses (Table 2). Moreover, CTB enhanced IL-1 secretion from KLH-stimulated SAC (Table 3). These findings suggest that CTB acts on APC to exert an immunoenhancing effect. Recently, whole CT has been shown to enhance antigen presentation by macrophages *in vitro*.[22] The present study is the first to report that CTB potentiates APC function. On the other hand, CTB is known to modulate proliferation and differentiation of T cells and B cells *in vitro*.[13-15,21] It has been demonstrated that the use of CTB or a CT/CTB mixture as an adjuvant *in vivo* causes enhanced induction of antigen-specific memory B cells[23] or T cells.[24] The effects of CTB on T cells and B cells might also be involved in its immunoenhancement. *In vitro* studies focusing upon T cells and B cells are in progress.

These *in vitro* data may help us to understand the mechanisms by which CTB acts as an adjuvant *in vivo*. The *in vivo* adjuvant effect was exerted only when CTB and antigen were given to mice at the same time and via the same route,[25] suggesting that CTB acts on early events in the antibody responses at the administration site. This is consistent with the present *in vitro* results. Further studies are in progress to determine whether the adjuvant action of CTB observed in nasal[9,10] or oral immunization[26] involves mucosal APC in the respiratory tract or intestine. Apart from this, our colleagues have recently demonstrated that CTB increases mucosal permeability for other proteins, using an Ussing chamber system, an *in vitro* model of mucosal drug absorption[27,28] (S. Gizurarson, submitted for publication). The *in vivo* adjuvant action of CTB might be achieved by a combination of its direct effects on the cells of the immune system such as APC, and its diverse effects on other cells and tissues such as mucosa.

Whole CT is widely known to enhance antibody responses to other antigens both *in vitro*[29,30] and *in vivo*[4-8]. The immunoenhancing effect of CT has been ascribed to its capacity to elevate intracellular cAMP levels,[1-3] because the enhancing effect was seen *in vitro* not only with CT but also with various cAMP-elevating agents, e.g. aminophylline, isobutylmethylxanthine, isoproterenol, prostaglandin E_1 and cAMP analogues.[29-32] However, the present study indicates that CTB, lacking cAMP-elevating capacity,[1,2] does have an immunoenhancing effect *in vitro*. In this culture system, CT was approximately 20-fold more effective on a molar basis than CTB in enhancing anti-KLH antibody responses (Y. Hirabayashi, S. Tamura, K. Shimada and T. Kurata, unpublished data). Taken together, the *in vitro* immunoenhancing effect of CT appears to be exerted by two different mechanisms which may work synergistically, i.e. cAMP-dependent and independent mechanisms mediated by the A subunit and the B subunit respectively, is consistent with *in vivo* results.[33] Further analyses are required to elucidate these mechanisms.

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REFERENCES

1. Holmgren J. (1981) Actions of cholera toxin and the prevention and treatment of cholera. *Nature*, **292**, 413.
2. Van Heyningen S. (1982) Cholera toxin. *Biosci. Rep.* **2**, 135.

3. Moss J., Burns D.L., Hsia J.A., Hewlett E.L., Guerrant R.L. & Vaughn M. (1984) Cyclic nucleotides: mediators of bacterial toxin action in disease. *Ann. Intern. Med.* **101,** 653.
4. Northrup R.S. & Fauci A.S. (1972) Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cells. *J. infect. Dis.* **125,** 672.
5. Kateley J.R., Kasarov L. & Friedman H. (1975) Modulation of *in vivo* antibody responses by cholera toxin. *J. Immunol.* **114,** 81.
6. Elson C.O. & Ealding W. (1984) Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* **132,** 2736.
7. Lycke N. & Holmgren J.(1986) Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. *Immunology*, **59,** 301.
8. Wilson A.D., Stokes C.R. & Bourne F.J. (1989) Adjuvant effect of cholera toxin on the mucosal immune response to soluble proteins: differences between mouse strains and protein antigens. *Scand. J. Immunol.* **29,** 739.
9. Tamura S.-I., Samegai Y., Kurata H., Nagamine T., Aizawa C. & Kurata T. (1988) Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. *Vaccine*, **6,** 409.
10. Hirabayashi Y., Tamura S.-I., Suzuki Y., Nagamine T., Aizawa C., Shimada K. & Kurata T. (1991) H-2-unrestricted adjuvant effect of cholera toxin B subunit on murine antibody responses to influenza virus haemagglutinin. *Immunology*, **72,** 329.
11. McKenzie S.J. & Halsey J.F. (1984) Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *J. Immunol.* **133,** 1818.
12. Van Der Heijden P.J., Bianchi A.T.J., Dol M., Pals J.W., Stok W. & Bokhout B.A. (1991) Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice. *Immunology*, **72,** 89.
13. Woogen S.D., Ealding W. & Elson C.O. (1987) Inhibition of murine lymphocyte proliferation by the B subunit of cholera toxin. *J. Immunol.* **139,** 3764.
14. Lycke N. & Strober W. (1989) Cholera toxin promotes B cell isotype differentiation. *J. Immunol.* **142,** 3781.
15. Spiegel S., Fishman P.H. & Weber R.J. (1985) Direct evidence that endogenous GM_1 ganglioside can mediate thymocyte proliferation. *Science*, **230,** 1285.
16. Elson C.O., Ealding W. & Lefkowitz J. (1984) A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. *J. immunol. Meth.* **67,** 101.
17. Kumagai K., Itoh K., Hinuma S. & Tada M. (1979) Pretreatment of plastic petri dishes with fetal calf serum: a simple method for macrophage isolation. *J. immunol. Meth.* **29,** 17.
18. Hodes R.J. & Singer A. (1977) Cellular and genetic control of antibody responses *in vitro*. I. Cellular requirements for the generation of genetically controlled primary IgM responses to soluble antigens. *Eur. J. Immunol.* **7,** 892.
19. Gillis S. & Mizel S.B. (1981) T-cell lymphoma model for the analysis of interleukin 1-mediated T-cell activation. *Proc. natl. Acad. Sci. U.S.A.*, **78,** 1133.
20. Denizot F. & Lang R. (1986) Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. immunol. Meth.* **89,** 271.
21. Elson C.O. & Solomon S. (1990) Activation of cholera toxin-specific T cells *in vitro*. *Infect. Immun.* **58,** 3711.
22. Bromander A., Holmgren J. & Lycke N. (1991) Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages *in vitro*. *J. Immunol.* **146,** 2908.
23. Chen K.-S. & Strober W. (1990) Cholera holotoxin and its B subunit enhance Peyer's patch B cell responses induced by orally administered influenza virus: disproportionate cholera toxin enhancement of the IgA B cell response. *Eur. J. Immunol.* **20,** 433.
24. Clarke C.J., Wilson A.D., Williams N.A. & Stokes C.R. (1991) Mucosal priming of T-lymphocyte responses to fed protein antigens using cholera toxin as an adjuvant. *Immunology*, **72,** 323.
25. Tamura S.-I., Samegai Y., Kurata H., Kikuta K., Nagamine T., Aizawa C. & Kurata T. (1989) Enhancement of protective antibody responses by cholera toxin B subunit inoculated intranasally with influenza vaccine. *Vaccine*, **7,** 257.
26. Hirabayashi Y., Kurata H., Funato H., Nagamine T., Aizawa C., Tamura S.-I., Shimada K. & Kurata T. (1990) Comparison of intranasal inoculation of influenza HA vaccine combined with cholera toxin B subunit with oral or parenteral vaccination. *Vaccine*, **8,** 243.
27. Ussing H.H. & Zerhan K. (1951) Active transport of sodium as the source of electrical current in the short-circuited isolated frog skin. *Acta physiol. Scand.* **23,** 110.
28. Wheatley M.A., Dent J., Wheeldon E.B. & Smith P.L. (1988) Nasal drug delivery: an *in vitro* characterization of transepithelial electrical properties and fluxes in the presence or absence of enhancers. *J. Contr. Rel.* **8,** 167.
29. Cook R.G., Stavitsky A.B. & Schoenberg M.D. (1975) Regulation of the *in vitro* early anamnestic antibody response by exogenous cholera enterotoxin and cyclic AMP. *J. Immunol.* **114,** 426.
30. Burchiel S.W. & Melmon K.L. (1979) Augmentation of the *in vitro* humoral immune response by pharmacologic agents. I. An explanation for the differential enhancement of humoral immunity via agents that elevate cAMP. *Immunopharmacology*, **1,** 137.
31. Ishizuka M., Braun W. & Matsumoto T. (1971) Cyclic AMP and immune responses. I. Influence of poly A : U and cAMP on antibody formation *in vitro*. *J. Immunol.* **107,** 1027.
32. Teh H.-S. & Paetkau V. (1974) Biphasic effect of cyclic AMP on an immune response. *Nature*, **250,** 505.
33. Wilson A.D., Clarke C.J. & Stokes C.R. (1990) Whole cholera toxin and B subunit act synergistically as an adjuvant for the mucosal immune response of mice to keyhole limpet haemocyanin. *Scand. J. Immunol.* **31,** 443.