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Cholera toxin B subunit (CTB) entrapped in microparticles shows comparable immunogenicity to CTB mixed with whole cholera toxin following oral immunization

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Abstract

Cholera toxin B subunit (CTB) was entrapped in poly(lactide-co-glycolide) microparticles and the responses induced by microencapsulated CTB were comparable to those induced by oral immunization with CTB mixed with whole cholera toxin. In addition, the CTB was released from microparticles intact following in vitro incubation, as determined by the ability of the CTB to bind to its cellular receptor, GM1. Moreover, microencapsulated CTB induced serum antibodies against intact CTB and not against monomeric fragments.

Keywords: Cholera toxin; Microparticle; Poly(lactide-co-glycolide); Vaccine; Antigen integrity

Several recent publications have highlighted the potential of poly(lactide-co-glycolide) microparticles as oral antigen delivery systems and as potential single dose vaccines (O'Hagan, 1994). A recent paper described the ability of microparticles to induce potent antibody and cell mediated immune responses following oral immunization and compared the responses to those induced by ISCOMS (Maloy et al., 1994). Nevertheless, few studies have compared the relative immunogenicity of novel delivery systems such as microparticles with alternative adjuvants or carrier systems. In a previous study (O'Hagan et al., 1993), the ability of microparticles to orally deliver CTB was evaluated. However, only the serum immune responses were determined and secretory immunity was not evaluated. In the present study, the immunogenicity of CTB entrapped in microparticles was compared to that of CTB mixed with whole cholera toxin (CT), which is the most potent oral adjuvant available (Czerkinsky et al., 1989) and both the serum and intestinal antibody responses were determined in mice.

One potential concern in relation to microencapsulation technology is the effect of the microparticle preparation process on the integrity of encapsulated antigens. The most commonly used preparation process for poly(lactide-co-glycolide) microparticles involves solvent evaporation, and

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during this process, the antigen is exposed to organic solvents, high shear and freeze-drying (Jeffery et al., 1993). Consequently, there is concern that labile molecules may be adversely affected during the microencapsulation process. To address this issue, we have investigated the antigenicity and immunogenicity of the CTB entrapped in microparticles.

CT is an enterotoxin produced by *Vibrio cholerae*, which consists of an A subunit and five B subunits held together in a pentamer (Holmgren, 1981). The enterotoxic effects of CT are caused by the A subunit, which activates adenylate cyclase in epithelial cells, increasing intracellular cyclic AMP and interfering with ion transport (Holmgren, 1981). The five B subunits, each of relative molecular mass of about 11 600 Da, form a complex pentameric structure, which binds to the ganglioside GM1 receptor, which is found on most mammalian cells (Holmgren, 1981). Hence, CTB functions as a targeting agent for the enterotoxic subunit A of CT. CTB is a particularly useful molecule with which to investigate the effects of microencapsulation, since it possesses a complex structure which is crucial for receptor binding. Moreover, the pentamer has been shown to dissociate and to lose immunogenicity at pH < 4 (Clements et al., 1986). For this reason, CTB is normally administered to humans in bicarbonate buffer to protect it against gastric acidity (Clements et al., 1986).

Microparticles with entrapped CTB, which was produced in *V. cholerae*, strain JS1569, as previously described (Sanchez and Holmgren, 1989; Lebens et al., 1993), were prepared using a 50:50 DL-lactide/glycolide co-polymer (RG 506 from Boehringer Ingelheim, Germany). The microparticles were prepared using a water-in-oil-in-water emulsion solvent evaporation process as previously described and were extensively washed (Jeffery et al., 1993). The microparticles were sized using photon correlation spectroscopy and had a mean size of 0.42 um (Malvern Instruments, UK). The loading level of the microparticles and the efficiency of encapsulation of CTB were determined following overnight dissolution of the microparticles in 5% SDS/NaOH, as previously described (Jeffery et al., 1993). The microparticles contained 3.0% w/w of entrapped CTB and the entrapment efficiency was 95%. Hence, 9.5 mg of a 10 mg batch of CTB used to prepare microparticles was entrapped in the microparticles.

To release the entrapped CTB, 10 mg of microparticles were suspended in 4 ml phosphate buffer (0.01 M, pH 8.0) and incubated for 72 h with constant rotation to allow in vitro release of the entrapped CTB. About 1 μg of the CTB was then subjected to SDS-PAGE (Lebens et al., 1993) on a Phastgel (Pharmacia, Sweden) alongside native CTB, and the gel bands were stained with Coomassie blue. SDS-PAGE analysis of CTB confirmed our previous observation that CTB released from microparticles showed the same bands as native CTB (O'Hagan et al., 1993).

Following in vitro release of CTB as described above, 1.5 ml of the suspension was centrifuged to remove particles and the ability of the released CTB to bind to the GM1 receptor was determined in an ELISA, as previously described (Czerkinsky et al., 1989). Quantitatively, the GM1 ELISA showed that after 72 h, 100 μg of CTB was present in the supernatant. Since the loading level of the microparticles was 3.0% w/w and 10 mg of microparticles was suspended in 4 ml of buffer, 100 μg of CTB in 1.5 ml represents release of about 88% of the entrapped CTB. The remaining 2.5 ml of microparticle suspension was further incubated at 4° C for 5 months to allow complete hydrolysis of the polymer and release of the remaining entrapped CTB. After this time, a further 1.5 ml aliquot of the suspension was taken, spun down as before and the levels of intact CTB in the supernatant was determined in the GM1 ELISA. The ELISA indicated that the supernatant contained 120 μg of CTB. Therefore, within experimental error for the assay (total release of entrapped CTB should have been 112.5 μg) the CTB appeared to be released intact from the microparticles.

To demonstrate the immunogenicity of the CTB entrapped in microparticles, groups of four to six C57BL/6 female mice (8-12 weeks old) were immunized with microparticles. One group of four mice were immunized intraperitoneally with 5 μg of CTB. The mice were boosted 2

weeks later with the same dose of CTB in microparticles, and serum samples were obtained from blood samples collected 1 week later. The serum IgG antibody titer following intraperitoneal immunization in mice with CTB in microparticles was 63 722 ± 25 846 ($n = 4$). For comparison, two mice were immunized with soluble CTB using the same dose and schedule. The serum IgG titers in these two mice were 19 236 and 21 346. Therefore, these studies indicated that CTB retained antigenicity following encapsulation in microparticles, and also that the immunogenicity may be enhanced.

To assess the integrity of CTB released from microparticles in vivo, the serum antibody responses to both pentameric CTB and to the monomeric units were determined using an adaptation of the GM1 ELISA. Antisera raised by intraperitoneal immunization with CTB in microparticles was investigated to determine the levels of antibodies against intact CTB pentamer and against monomers of CTB using a GM1 ELISA, modified as previously described (Hirst and Holmgren, 1987). Briefly, CTB was bound in a GMI ELISA and was then confirmed to be present as a pentamer by the monoclonal antibody LT39, which only recognises pentameric CTB and does not bind to monomers. For the ELISA to detect antibodies against monomeric fragments of CTB, the plates were coated with acid treated CTB, under conditions that will not allow re-assembly of CTB. As controls in this assay, LT39 was included, in addition to a second monoclonal antibody, CT6, which recognises both pentameric and monomeric CTB (Hirst and Holmgren, 1987). These assays showed that most of the antibodies were induced against the CTB pentamer and only low levels of antibodies were induced against the monomers. Hence, these studies confirmed that CTB in microparticles was intact and also showed that it was released from microparticles intact in vivo.

One group of mice were orally immunized with 20 μg of CTB entrapped in microparticles on three occasions separated by 2 week intervals. The response to CTB in microparticles was compared to that induced by a similar oral immunization schedule in a separate group of mice, with 20 μg of soluble CTB mixed with 1.5 μg of CT.

In addition, one group of mice were orally administered with 7 μg of CTB entrapped in microparticles per day, for three consecutive days, followed by a similar repeat schedule 4 weeks later. For comparison, an additional group of mice were immunized with 7 μg of soluble CTB administered in a similar schedule, mixed with 0.75 μg of CT per day.

Following oral immunization the antibody responses in serum and intestinal samples were determined by a novel ELISA (Czerkinsky and Holmgren, in preparation). To obtain the tissue samples, the mice were exsanguinated and perfused with heparinized phosphate-buffered saline (PBS). Samples of tissue (100–500 mg) were then removed and washed in PBS. The tissue samples were then homogenized and extracted into 100 μl of buffer per 100 mg of tissue and ELISA was performed on the extracts. The ELISA results are expressed as titer per 100 mg of tissue (full details of the ELISA technique are to be published separately by C. Czerkinsky and J. Holmgren).

Following oral immunization, the responses induced by microparticles with entrapped CTB were comparable to those induced by immunization with CTB mixed with CT. Fig. 1 shows the serum IgG antibody titers following oral immunization

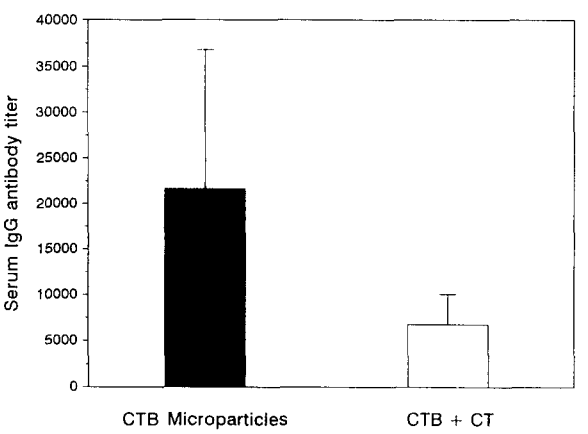

Fig. 1. Serum IgG antibody responses following oral immunization on three occasions, separated by 2 weeks, with CTB (20 μg) entrapped in microparticles or with CTB (20 μg) plus CT (1.5 μg).

on three occasions with CTB in microparticles and CTB with CT. An unpaired Student's t-test was used to compare the means and to assess statistical significance. The results were considered statistically significant if $p < 0.05$. There were no significant differences between the two groups. Fig. 2 shows the intestinal IgA antibody titers following repeated oral immunization over 3 days, on two separate occasions, with CTB in microparticles and CTB with CT. Again, there were no significant differences between the two groups.

In the current studies, the observations relating to the immunogenicity of CTB in microparticles were very encouraging. Following parenteral immunization, the immunogenicity of CTB entrapped in microparticles appeared to be enhanced. More importantly, CTB entrapped in microparticles was potently immunogenic following oral immunization. CTB in microparticles showed comparable immunogenicity to CTB mixed with CT, the most potent oral adjuvant available (Czerkinsky et al., 1989).

However, perhaps the most significant finding of the current studies relates to the maintenance of the antigenic integrity of CTB following encapsulation in PLG microparticles. Although several peptides and proteins have been successfully entrapped in microparticles with maintenance of biological activity, stability problems have been reported with others (Johnson et al., 1991). Peptides and proteins are immiscible with PLG polymers and consequently, do not tend to become molecularly dispersed within polymeric microparticles. Instead, peptides and proteins tend to exist as isolated domains within the polymeric network of the freeze-dried product. Although, in general, proteins in the solid state are more stable, entrapment of such materials in polymeric devices has previously resulted in aggregation problems (Brown et al., 1986). Nevertheless, in the current studies, CTB was entrapped in microparticles and released in vitro without evidence of aggregation. The SDS-PAGE findings in the current studies were supported by observations which showed that the released CTB was capable of interacting with its cellular receptor, GM1. Moreover, following intraperitoneal immunization in mice, the microencapsulated CTB was shown to induce antibodies against the intact pentamer, with minimal antibodies against the monomer units. Hence, the current studies showed that CTB was released from microparticles intact, both in vitro and in vivo. Although it may be possible that CTB could have re-combined into a pentamer following release in vitro, this interpretation seems unlikely. The in vivo data confirm that the CTB was released intact from the microparticles and recombination into a pentameric structure would have been extremely unlikely in the complex environment of the peritoneal cavity.

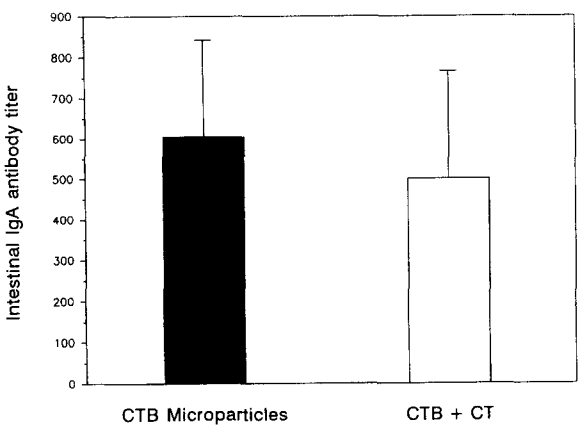

Fig. 2. Intestinal IgA antibody responses following oral immunization on three consecutive days, on two occasions separated by 4 weeks, with CTB (7 μg per day) entrapped in microparticles or with CTB (7 μg per day) plus CT (0.75 μg per day).

CTB was an attractive molecule to use in the current studies because of its complex structure, and also because it has been reported to be unstable at low pH. PLG is a polyester, which degrades in vitro and in vivo to produce lactic and glycolic acids. For this reason, it has been suggested that PLG delivery systems are only suitable for the controlled release of acid stable agents (Hutchinson, 1988). The data from the present studies would indicate that this interpretation is perhaps an over-simplification and may not apply to all 'acid-unstable' molecules.

In conclusion, the current studies indicated that a soluble, acid unstable, recombinant molecule with a complex structure, CTB, could

be entrapped in PLG microparticles and released intact, both in vitro and in vivo. Moreover, the entrapped CTB was potently immunogenic following both parenteral and oral immunization. These studies offer considerable promise for the future development of improved vaccines through microencapsulation of recombinant antigens.

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