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Manipulation of intestinal immune responses against ovalbumin by cholera toxin and its B subunit in mice

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SUMMARY

We studied the effect of mucosal presentation of ovalbumin (OVA) conjugated to cholera toxin (CT) or cholera toxin B subunit (CTB) on the intestinal immune responses against OVA. Mice were primed intraperitoneally (i.p.) with OVA in a water-in-oil emulsion and boosted intraduodenally (i.d.) with OVA conjugated to CT or CTB in various molar ratios. Responses were evaluated by testing intestinal secretions for OVA-specific antibodies and by quantifying the OVA-specific antibody secreting cells (ASC) in the lamina propria of the small intestine. OVA–CT conjugates were tested in a molar ratio ranging from 1·8:1 to 4500:1. OVA–CTB conjugates were tested in a molar ratio ranging from 0·25:1 to 500:1. The optimum intestinal immune response was reached at a molar ratio of 1·8:1 for OVA–CT and 5:1 for OVA–CTB. The binding capacity of OVA–CTB, but not of OVA–CT, to GM1 ganglioside corresponded with the capacity to enhance the intestinal immune response. The effect of conjugating CTB or CT to OVA on the immune response against OVA was more striking when mice were not only boosted i.d., but also primed i.d. Both OVA–CT and OVA–CTB induced detectable immune responses, whereas free OVA did not. Therefore, the carrier effect of CT or CTB is essential to trigger a mucosal immune response against OVA when presented mucosally only. We conclude that enhancing antigen uptake greatly facilitates mucosal immune responses.

INTRODUCTION

Most dead, non-replicating antigens do not induce mucosal immune responses after mucosal antigen presentation. Exceptions are cholera toxin (CT) and the heat-labile toxin of *Escherichia coli* (LT), which induce significant mucosal immune responses upon oral immunization.[1,2] This is due to the fact that both CT and LT bind actively to the intestinal epithelium by their B subunits via the GM1 ganglioside receptor and stimulate adenylcyclase by their A subunits.[3] Furthermore, CT and LT can provoke the induction of responses against non-related antigens administered orally together with CT or LT.[4–7] Although activation of adenylcyclase by the A subunit of CT or LT may be an important factor in the latter effect,[8,9] it has been suggested that enhanced antigen uptake through binding to the intestinal epithelium via the B subunit by itself will also lead to enhanced mucosal immune responses.[4] However, until now no correlation has been reported between binding to the epithelium and the stimulation of the mucosal immune response. Various authors have studied the non-toxic B subunits from CT and LT (CTB and LTB) as possible carrier proteins for mucosal antigen presentation. Unfortunately, the results obtained so far are not conclusive. CTB has been found to stimulate mucosal responses when given simultaneously with non-related antigen.[10,11] Others described that CTB must be conjugated with the antigen to be effective.[12,13] In contrast, Liang, Lamm & Nedrud[14] and Lycke & Holmgren[7] reported that CTB, either conjugated or not, does not stimulate mucosal responses. LTB has been found to be ineffective when given simultaneously with the antigen,[6] but effective when presented as a fusion protein with the antigen.[15]

In this paper we studied in detail the effect of conjugating CT and CTB to ovalbumin (OVA) upon the mucosal immune response against OVA in various immunization regimens. Furthermore, we studied whether these effects correlated with the binding of the conjugates to GM1 ganglioside.

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MATERIALS AND METHODS

Mice

Female C3H/He mice were purchased from Harlan-Olac Ltd, Bicester, Oxon, U.K., housed under conventional circumstances, and used at the age of 12–20 weeks.

Antigens

Ovalbumin (OVA; Sigma, St Louis, MO) was conjugated to CTB (Sigma) or CT (Sigma) by glutaraldehyde (Merck, Darmstadt, Germany) in various molar ratios. In short, OVA and CTB or OVA and CT were dissolved in 0·01 M phosphate-

buffered saline (PBS; pH 8·0). Glutaraldehyde was slowly added to the mixtures until a concentration of 15 mM was reached. After 1 hr of gently stirring, the reactions were terminated by adding excess glycine (60 mM). The resulting mixtures, containing OVA–CTB and OVA–CT, were dialysed against PBS (pH 8·0). Polymerized OVA (pOVA) was prepared as described earlier.[16]

GM1-ELISA

OVA–CT and OVA–CTB conjugates were tested for their ability to bind to ELISA plates coated with GM1 ganglioside (Sigma). Detection of bound CT and CTB was performed according to the method described by Svennerholm & Holmgren.[17] Rabbit anti-CT–peroxidase conjugate (RaCT/PO; a kind gift from Dr F.G. Van Zijderveld, CVI, Lelystad, The Netherlands) was used to detect bound CT or CTB. Bound OVA was detected by adding mouse anti-ovalbumin (MaOVA; CVI) followed by sheep anti-mouse Ig–peroxidase conjugate (ShaMIgGPO; Serotec, Kidlington, Oxon, U.K.). Bound peroxidase conjugates were made visible by adding 3,3,5,5,-tetramethylbenzidin (Boehringer, Mannheim, Germany) and H_2O_2 as a colouring substrate. After incubation for 10 min, the reaction was stopped by adding 100 μl of 0·1 N H_2SO_4. Absorbance was measured at 450 nm.

Immunizations

Mice were injected intraperitoneally (i.p.) with 0·1 ml of a water in oil (W/O) emulsion,[18] containing 0·1 mg pOVA. Four weeks later the mice were given booster immunizations intraduodenally (i.d.) with 150 μg antigen in 0·5 ml 0·2 M $NaHCO_3$. To this end the mice were anaesthetized with Avertin (Aldrich, Brussels, Belgium),[19] the abdominal cavity opened and the antigen injected in the duodenum approximately 1 cm after the stomach. The incision was closed in two layers. As antigens were used pOVA, pOVA with free CTB (150 μg) or free CT (10 μg), OVA–CTB and OVA–CT.

In another series of experiments mice were primed i.d. with 150 μg of pOVA, OVA–CTB or OVA–CT. Four weeks later the mice were given booster immunizations i.d. with the same antigen preparations. In one separate experiment OVA and OVA–CTB immunized mice were boosted i.p. with 150 μg OVA 1 week after the second i.d. immunization.

Detection of anti-OVA antibodies in intestinal secretions

Intestinal secretions were obtained by scraping the isolated small intestine, as described in detail previously.[20] Briefly, small intestines were removed from the mice and flushed with PBS (pH 7·2). Mucus was squeezed out. The scrapings were dissolved in 2 ml PBS containing 50 mM EDTA and 0·1 mg/ml trypsin inhibitor and mixed vigorously. The solution was clarified by centrifugation (10 min, 650 *g*) and 10 μl NaN_3 were added before storage at −20°. Anti-OVA antibodies were assayed by ELISA on microtitre plates coated with pOVA, as described elsewhere.[16]

Detection of OVA-specific antibody-secreting cells (ASC)

Lymphocytes from the lamina propria of the small intestine were isolated as described elsewhere.[21] OVA-specific ASC were quantified by an ELISA-spot assay.[22]

Table 1. Anti-OVA IgA titres in intestinal secretions of mice primed with OVA i.p. and boosted with various antigen preparations i.d.

Antigen in booster	IgA titre ± SE in intestinal secretions
pOVA	23 ± 11
pOVA + CT	208 ± 85
OVA-CT	256 ± 114
pOVA + CTB	20 ± 12
OVA–CTB	87 ± 22

Titres are expressed as the mean ($n = 5$) with the SE.

RESULTS

Stimulation by CT and CTB

First it was studied whether CT and CTB could stimulate the intestinal IgA response against OVA in an i.d. booster after an i.p. priming of OVA in a W/O emulsion. Mice were boosted i.d. with pOVA, pOVA + free CT, OVA–CT (molar ratio 1·8:1), pOVA + free CTB, or OVA–CTB (molar ratio 0·25:1). Eight days after booster immunization intestinal secretions were collected and tested for anti-OVA IgA by ELISA. The results clearly demonstrate (Table 1) that CT stimulated the response to OVA regardless of whether it was conjugated or not. CTB stimulated the response only when conjugated to the antigen.

Binding of OVA–CT and OVA–CTB to GM1

OVA was conjugated to CT in molar ratios varying from 1·8:1 to 4500:1 or conjugated to CTB in molar ratios varying from 0·25:1 to 500:1. The capacity to bind to GM1 ganglioside of the conjugated products was examined by ELISA on GM1-coated microtitre plates. Bound antigen was measured after addition of MaOVA followed by ShaMIgG/PO. The absorbance measured was optimal for OVA–CT at a molar ratio of 18:1 to 45:1 and for OVA–CTB at a molar ratio of 1:1 to 5:1 (Fig. 1).

When RaCT/PO was used as detecting agent, the conjugates with the highest CT or CTB input (molar ratio OVA:CT 1·8:1 and OVA:CTB 0·25:1) reached the highest absorbances (Fig. 1). The conjugates with the lowest molar ratio (4500:1 for OVA–CT and 500:1 for OVA–CTB) reached the lowest absorbances, which were, however, still above control level. When free pOVA was used as antigen, no signal was observed in the assay with either RaCT or MaOVA as detecting antiserum (results not shown).

Effect of conjugating CT or CTB to OVA on the OVA-specific immune response in intestinal secretions

In two separate experiments mice were immunized i.p. with OVA in W/O emulsion and boosted i.d. with OVA–CT conjugates in various molar ratios or with OVA–CTB conjugates in various molar ratios. As a control other mice were boosted i.d. with pOVA. The intestines of mice were removed and intestinal secretions collected by scraping 8 days after booster immunization. The samples were assayed for the

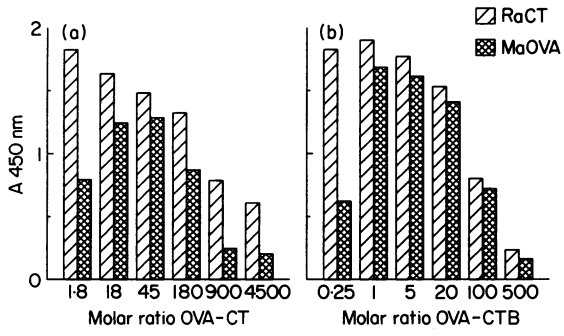

Figure 1. Binding of OVA–CT (a) and OVA–CTB (b) conjugates to GM1-coated ELISA plates detected by MaOVA/ShaMIgGPO. As a control bound CT and CTB were detected by RaCT. Results are expressed as the absorption at 450 nm at an antigen–carrier dilution of 0·02 mg/ml.

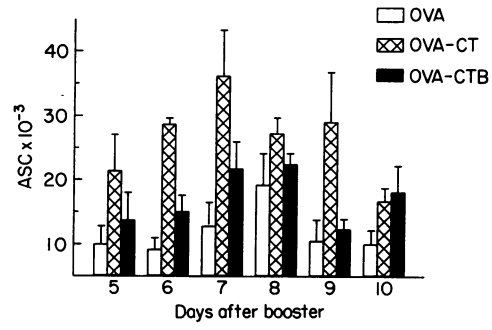

Figure 3. Number of OVA-specific IgA–ASC in the small intestine after i.d. booster immunization with OVA, OVA–CT or OVA–CTB. Results are expressed as the mean (n = 5–10) with the SE shown in bars. Molar ratios of the antigen–carrier conjugates were 2·5:1 for OVA–CTB and 18·1 for OVA–CT.

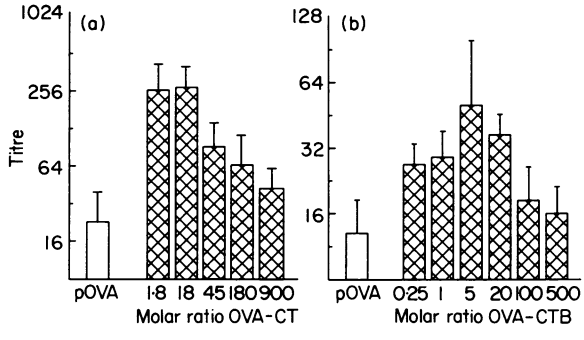

Figure 2. Effect of CT (a) and CTB (b) coupled in various molar ratios to OVA on the intestinal immune response measured by ELISA in intestinal scrapings 8 days after i.d. booster immunization. Results are expressed as the mean titre ($n = 10$) with the SE shown in bars.

presence of OVA-specific antibodies by ELISA. OVA–CT stimulated the response best when conjugated at a molar ratio of 1·8:1 or 18:1 (Fig. 2). At higher molar ratios the stimulation decreased. OVA–CTB stimulated the response best when conjugated in a molar ratio of 5:1. At higher molar ratios the stimulatory effect decreased gradually. The shape of the curve of intestinal responses after i.d. booster immunization with OVA–CTB conjugated in various molar ratios (Fig. 2b) corresponded with the shape of the curve of the binding capacity of the OVA–CTB conjugates to GM1 detected by MaOVA (Fig. 1b). This indicates that the binding capacity of OVA–CTB to GM1 correlated with the ability to stimulate the intestinal immune response.

Effect of conjugating CT or CTB to OVA on the number of intestinal OVA-specific ASC

We quantified the number of OVA-specific ASC in the lamina propria of immunized mice to determine whether the enhanced mucosal immune responses were due to an enhanced secretion of IgA per cell or to an enhanced number of IgA secreting cells in the lamina propria. Mice were immunized i.p. with OVA in W/O emulsion and boosted i.d. with OVA–CT (molar ratio 18:1) or OVA–CTB (molar ratio 5:1). Lamina propria lymphocytes from the small intestines were isolated 5,6,7,8,9 and 10 days after booster immunization and the number of OVA-specific ASC measured by ELISA spot assay. The responses were compared with the response detected after booster immunization with free pOVA. Conjugating CTB stimulated the response 1·5–2 times, whereas conjugating CT stimulated the response 2·5–3·5 times (Fig. 3). The kinetics of the responses after immunization with OVA–CTB or OVA–CT did not differ compared with the kinetics after immunization with free pOVA.

OVA-specific IgG–ASC were detected in the lamina propria cell suspensions in low numbers. Maximum responses were detected 6 days after i.d. booster immunization: OVA-immunized mice had 2220 IgG–ASC per small intestine, OVA–CT immunized mice 4320 and OVA-CTB immunized mice 2880. OVA-specific IgM–ASC were not detected above background level.

Effect of conjugating CT or CTB with OVA on the immune response after i.d. priming and booster immunization

In the experiments described above mice were primed i.p. with OVA in W/O emulsion. In another series of experiments mice were primed i.d. with pOVA, OVA–CT (molar ratio 18:1) or OVA–CTB (molar ratio 5:1) and boosted i.d. with the same antigen preparations to study whether OVA–CT and OVA–CTB could trigger detectable immune responses upon mucosal antigen presentation. OVA-specific IgA antibodies were determined in intestinal secretions and in serum 7 days after booster immunization. All pOVA immunized mice did not react with detectable responses in intestinal secretions or in sera (Table 2). In contrast, several OVA–CT and OVA–CTB immunized mice responded with IgA titres in scrapings and IgG titres in sera. Responder mice immunized with OVA–CT gave higher IgA titres in scrapings than responder mice immunized with OVA–CTB.

To determine whether the non-responder mice were primed for an anamnestic systemic immune response or were rendered tolerant to OVA, in a separate experiment mice were primed and boosted i.d. with pOVA or OVA–CTB. Two weeks after the second i.d. immunization the mice were injected i.p. with 150 μg OVA. One week after booster immunization blood was collected and serum tested for the presence of anti-OVA antibodies.

Table 2. Anti-OVA antibody titres in scrapings and sera of mice i.d. primed and boosted with OVA, OVA-CT or OVA-CTB

Immunogen	Responder/ total no. of animals	IgA titre scrapings		IgG titre sera	
		Resp.	Non-resp.	Resp.	Non-resp.
OVA	0/10	—	≤2	—	≤50
OVA-CT	4/10	47·6	≤2	4600	≤50
OVA-CTB	3/10	20·0	≤2	8800	≤50

Titres are expressed as the mean of responder mice or non-responder mice.

Mice primed and boosted i.d. with OVA–CTB reacted to the i.p. injection (average titre 520±250), whereas mice immunized with free pOVA did not respond (average titre ≤50).

DISCUSSION

In this paper we studied the effect of conjugating CT or CTB to OVA on the i.d.-induced intestinal immune response against OVA. First we demonstrated that CT and CTB stimulated the intestinal immune response after an i.p. priming and an i.d. booster immunization (Table 1). Second, we confirmed earlier results[12] that conjugating CTB to the antigen is a pre-requisite to obtain the stimulatory effect, as free CTB did not stimulate the response above the level reached with pOVA alone. The stimulation by CT did not depend on conjugation.

It is thought that CTB can stimulate mucosal immune responses by facilitating the contact between the antigen and the intestinal epithelium. However, a direct correlation had not yet been demonstrated. We clearly demonstrated by ELISA that OVA–CTB binds to GM1 ganglioside (Fig. 1). The results obtained when MaOVA was used as detecting agent showed that the efficiency to bind to GM1 depended on the molar ratio between OVA and CTB. An optimum was reached at a molar ratio of 1:1–5:1. Probably, at these molar ratios there is an optimal combination of available GM1-binding sites and antigenic determinants that can be detected by MaOVA. At a molar ratio of 0·25:1 antigenic determinants of OVA were probably partly blocked by excess CTB, which resulted in a lower signal. At molar ratios higher than 5:1 the binding capacity of the OVA–CTB conjugates decreased. The absorbance reached when RaCT was used as detecting agent continuously declined from a molar ratio of 0·25:1 to 500:1 as a result of decreasing concentrations of CTB.

As OVA–CTB conjugates bind to GM1 ganglioside in an ELISA system, they probably also adhere to GM1 on intestinal epithelial cells. We examined whether or not the capacity to adhere to GM1 in ELISA correlated with the stimulatory effect on an i.d.-induced intestinal immune response. Indeed, the stimulation by CTB conjugated to OVA in various molar ratios (Fig. 2) corresponded with the binding capacity of the OVA–CTB conjugates in the GM1 ELISA (Fig. 1). As Bland & Warren[23] described that intestinal epithelial cells can function as antigen-presenting cells, active binding of antigen to these cells may result in enhanced immune responses.

In contrast to the effect observed using CTB, the stimulation by CT did not correlate with the binding of OVA–CT to GM1. The stimulation by CT showed a linear dose–response relationship that decreased from a molar ratio of 1·8:1 to 900:1, whereas binding to GM1 showed an optimum at molar ratios of 18:1 to 45:1. CT is known for its strong adjuvant properties provided by its A subunit.[24,25] Clearly, the adjuvant effect of the A subunit dominates the carrier effect of optimal binding of the B subunit to the intestinal epithelium.

To study the stimulatory effect of conjugating CT or CTB to OVA at the cellular level we measured the numbers of OVA-specific IgA–ASC in the small intestine of mice that were boosted with pOVA, OVA–CT or OVA–CTB. CTB stimulated the response 1·5–2 times, whereas CT stimulated the response 2·5–3·5 times (Fig. 3). CT and CTB enhanced the number of OVA-specific IgA-ASC in the small intestine somewhat less than the level of OVA-specific IgA antibodies in intestinal secretions. This indicates that the stimulation by CT and CTB is not only caused by an increase in the number of antigen-specific ASC in the lamina propria but also by an enhanced transport of IgA over the epithelium and/or an increased secretion of IgA by individual plasma cells.

The stimulation by CT and especially by CTB observed in our experiments (Table 1, Figs 2 and 3) is clear yet not very strong. This may be caused by the antigen used in our experiments, as Wilson *et al.*[25] reported that CT stimulated mucosal immune responses against OVA less than the response against keyhole limpet haemocyanin. More likely, it is due to the immunization regimen chosen for the experiments, since free pOVA is quite able to induce a mucosal immune response by itself.[16]

The effect of the carrier function of CT and CTB on the intestinal anti-OVA response was much more striking in an immunization regimen in which the antigen was presented to the mucosa twice via i.d. immunization (Table 2). Free OVA did not induce an immune response in this protocol. However, about half of the mice did show a detectable immune response upon immunization with OVA–CTB or OVA–CT. The percentage of responder mice can probably be increased by presenting the antigen to the mucosa more often, as Andre *et al.*[26] and Wachsmann *et al.*[27] stated that at least four oral immunizations are needed to induce mucosal immune responses. As we immunized the mice i.d. it was technically impossible to immunize the animals repeatedly on four occasions.

I.p. booster immunization with OVA revealed that mice i.d. immunized twice with OVA–CTB were primed for systemic response, whereas mice immunized i.d. twice with free pOVA were not. It is known that oral presentation of protein antigens usually results in the induction of a state of tolerance.[28,29] Although our experiments were not designed to study tolerance induction, the results indicate that conjugating CTB to OVA can abrogate tolerance induction. These results confirm the conclusions reported by Elson & Ealding,[4] but are in contrast to the conclusions reported by Clements *et al.*[6] and Lycke & Holmgren.[7] They suggested that the lack of tolerance induction by CT or CTB may be a function of binding to cell-surface receptors, while the ability to influence the immunological response to a second antigen may reside with the A subunit of the toxin.

The results presented in this paper show that a carrier protein like CTB greatly facilitates a mucosal SIgA response and decreases the chance of tolerance induction by antigens that do not induce a mucosal immune response by themselves. The

effect of a carrier protein is smaller in mice that are already primed for a mucosal immune response by another protocol (i.p. immunization of OVA in a W/O emulsion). In this situation whole CT still stimulates the mucosal immune response as a result of the activity of the A subunit. So, in agreement with Liang *et al.*[30] we conclude that the carrier function of CTB enhances the immunogenicity of mucosally presented antigen, whereas the A subunit of CT provides adjuvant activity.

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