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ARTICLE *in* VACCINE · JULY 2001

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Mucosal immunization of mice using CpG DNA and/or mutants of the heat-labile enterotoxin of *Escherichia coli* as adjuvants

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Received 30 November 2000; received in revised form 13 February 2001; accepted 16 February 2001

Abstract

Cholera toxin (CT) and the *Escherichia coli* heat-labile enterotoxin (LT) are potent mucosal adjuvants in animals associated, at least in part, with their ability to induce cAMP. While toxicity generally precludes their use in humans, a number of different subunit or genetically detoxified mutants of CT and LT have been developed. Another type of adjuvant that has been shown to be effective at mucosal surfaces comprises synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG ODN). We have previously demonstrated a synergy between CpG ODN and native toxins after intranasal (IN) administration to mice, and herein have examined whether this synergy is linked to the cAMP activity. The adjuvanticity of CpG ODN was evaluated with IN and oral delivery of tetanus toxoid or the hepatitis B surface antigen, relative to and in combination with native LT holotoxin (LTh), three active site mutants (LTS61F, LTA69G, LTE112K), a protease site mutant (LTR192G), and the B subunit of LT (LTB). At an equivalent dose, the adjuvants could generally be divided into two groups: one that included CpG ODN, LTh, LTR192G, and LTA69G which acted as strong adjuvants; and the second which comprised LTB, LTS61F, and LTE112K, which produced significantly weaker immune responses. When CpG ODN was co-administered with bacterial toxin-derivatives, in most cases, no synergy between CpG and the LT derivatives was found for strength of the humoral response. Nevertheless, for both routes and antigens, CpG ODN combined with any LT derivative induced a more Type 1-like response than LT derivative alone. These results suggest that while the synergy seen previously with native toxins may have been due in part to inherent cAMP activity, it may have also depended on the particular antigen used and the route of immunization.

Keywords: Mucosal; Adjuvant; CpG

1. Introduction

The majority of infectious diseases are transmitted through the mucosal surfaces of the gastrointestinal, genitourinary and respiratory tracts, yet most vaccines are given parenterally (i.e., by intramuscular (IM), subcutaneous or intradermal injection). The development of vaccines that could be delivered mucosally would help promote mucosal immune responses, as well as providing a safe, readily acceptable method for inducing systemic immune responses. One of the major setbacks in the development of mucosal vaccines has been the lack of a safe yet effective mucosal adjuvant for use in humans. A number of mucosal adjuvants have been proposed including bacterial toxins such as cholera toxin (CT) and *Escherichia coli* heat labile enterotoxin (LT) and their derivatives [1–7], lipid A derivatives [8,9], muramyl peptide derivatives [10–13], aluminum salts [14,15], saponin derivatives such as QS21 [16,17] and synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs (CpG ODN) [18–21].

In animal models, the most commonly used mucosal adjuvants are bacterial toxins, such as CT or the closely

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0264-410X/01/$ - see front matter
PII: S0264-410X(01)00088-3

related *E. coli* heat-labile LT. Both CT and LT are composed of one enzymatically active A chain and five B chains that bind to ganglioside GM1 expressed on the eukaryotic cell surface. Thereafter, the A subunit is proteolytically cleaved and reduced at its single disulphide bond to yield an enzymatically active A1 subunit and a smaller A2 peptide. The A1 subunit possesses both NAD-glycohydrolase and ADP-ribosyltransferase activity and binds to NAD, splitting it and transferring ADP-ribose to the regulatory GTPase Gsα of the adenylate cyclase system, permanently activating it to catalyze the formation of cAMP [22,23]. The exact mechanisms of CT and LT mucosal adjuvant activity are unknown; however, available evidence suggest that they are linked, at least partially, to ADP-ribosyltransferase activity [24].

Since both CT and LT are considered too toxic for human use, two strategies have been used to avoid the toxicity of the native toxins. The first strategy is use of the non-toxic B subunits that lack enzymatic activity [25,26]. The non-toxic B subunit has been used as adjuvant, with mixed results [1,2,24–27]. However, these findings have been somewhat complicated since, in some cases, the purified preparations of CTB used were subsequently found to contain some holotoxin contamination [28,29]. The second is the development of genetically detoxified mutants of CT or LT that have varying levels of residual enzymatic activity. These include active site mutants, such as R7K, S61F, S63K, A69G or E112K, whereby single amino acid mutations result in loss or reduction of ADP-ribosyltransferase activity and associated enterotoxicity [2,6,7], and protease site mutants, such as R192G, in which a single amino acid substitution within the disulphide bond results in resistance to proteolytic activation [3,4,6]. Each of these retains some or all of the adjuvant activity of the holotoxin but with varying degrees of residual toxicity.

The adjuvant activity of CpG ODN is due to several different effects it has on innate and adaptive immune responses. First, it causes B cells to proliferate and secrete immunoglobulin, and these direct effects synergize with antigen-specific effects mediated through the B cell receptor [30–32]. As well, CpG ODN causes upregulation of co-stimulatory molecules and MHC class II molecules, improving antigen presentation. CpG ODN also directly activates monocytes, macrophages and dendritic cells to secrete IFN-α/β, IL-6, IL-12, GM-CSF, chemokines, and TNF-α [31,33], which in turn stimulate T cells to secrete additional cytokines and natural killer (NK) cells to secrete IFN-γ [31,32,34–37]. A T-helper function is provided by the strong Type 1-like pattern of cytokine production that is dominated by IL-12 and IFN-γ, with little secretion of Type 2 cytokines [31].

CpG ODN is effective as a mucosal adjuvant with intranasally (IN) delivered antigen [18–21], and was found to be as potent an adjuvant as CT for induction of serum IgG and mucosal IgA [18,19]. Furthermore, there appeared to be a synergistic response when CpG ODN and CT but not CTB were combined [18,19,38], indicating that the synergy may have been associated with the cAMP activity of the CT. If so, it is possible that CpG ODN could be combined with genetically detoxified mutants that retain some enzymatic activity to produce improved adjuvant activity without additional toxicity. We have evaluated this possibility in the present study, using IN or oral delivery to mice of hepatitis B surface antigen (HBsAg) and tetanus toxoid (TT) with native LT (LTh), the B subunit of LT (LTB), various active site mutants (LTE112K, LTA69G, LTS61F) and a protease site mutant of LT (LTR192G).

2. Materials and methods

2.1. Immunization of mice with HBsAg or TT

Groups ($n = 5$) of female BALB/c mice (6–8 weeks, Charles River, Montreal, QC) were immunized with 10 μg HBsAg (plasma-derived HBV S protein, *ad* subtype, Genzyme Diagnostics, San Carlos, CA) or TT (formalin-inactivated TT, Aventis Pasteur, Swiftwater, PA), alone or combined with 1 or 10 μg of CpG ODN and/or LT, LTB or mutant LT. CpG ODN was made with a nuclease-resistant phosphorothioate backbone and had sequence (1826-TCCATGACGTTCCTGACGTT) (Coley Pharmaceutical Group, Wellesley, MA). We have previously shown this ODN to induce strong immune responses in mice after IM and IN administration and that this was due to the CpG motif rather than a non-specific effect of the ODN backbone [18,39]. LT-based adjuvants were constructed and purified as previously described [6], and were as follows: LTh, LTB, active site mutants LTS61F, LTA69G, LTE112K, and LTR192G. The LT derivatives have previously been shown to differ in their ability to induce accumulation of cAMP in cultured Caco-2 cells, such that LTB, LTE112K, LTS61F, LTR192G, LTA69G and LTh induced 0, 5, 2, 77, 323, and 775 pmole/ml cAMP respectively [6]. For IN immunization, vaccine solutions were made up to a total volume of 20 μl with 0.15 M NaCl and applied as droplets to the external nares under light anesthesia with Halothane® (Halocarbon Laboratories, River Edge, NJ). Mice were boosted in an identical manner at 4 and 8 weeks post prime. For oral immunization, vaccine solutions were made up to a total volume of 50 μl and administered using a 1 c.c. tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) attached to a 20-gauge olive tip steel feeding tube (Fine Science Tools

Inc., North Vancouver, BC), which was passed down the oral cavity and the esophagus, and into the stomach, also under light anesthesia. These mice were boosted in an identical manner at 7 and 14 days post prime. A different immunization schedule was chosen for oral immunization since we have previously found this to yield better immune responses in our laboratory (McCluskie and Davis, unpublished data).

2.2. Collection of samples

All samples were collected over a two-day period which, for IN and orally immunized mice, was 4 and 1 week, respectively, after the third and final immunization. Plasma was collected and lung washes carried out as previously described [18]. Vaginal secretion samples were collected by washing the vaginal cavity three times with 75 μl (225 μl total) of PBS containing 0.1 μg sodium azide (Sigma, St. Louis, MO). Saliva was obtained following i.p. injection with 100 μl of 1 mg/ml pilocarpine (Sigma) in PBS to induce saliva flow. Gut washes were obtained by removing the small intestine and passing 200 μl PBS containing 0.1% sodium azide through each of three 10 cm sections. For lung and gut washes, samples were stored for individual mice. For saliva and vaginal washes, samples were pooled for each group ($n = 5$). All samples were stored at -20°C until assayed by ELISA.

2.3. Evaluation of immune responses

HBsAg- and TT-specific antibodies (anti-HBs and anti-TT respectively) in the individual samples were detected and quantified by end-point dilution ELISA assay as described previously for IgG, IgG1, IgG2a isotypes [18] and total IgA [39]. End-point dilution titers for IgG isotypes in plasma and IgA in mucosal samples were defined as the highest sample dilution that resulted in an absorbance value (OD 450) at least twice that of a pooled non-immune plasma or mucosal sample following subtraction of background value. Anti-TT or anti-HBs titers for a group of animals were expressed as geometric mean titers ± the standard error of the mean (GMT ± SEM) of individual animal values, which were themselves the average of triplicate assays.

For mice immunized with HBsAg, CTL activity of splenocytes taken from mice 4 weeks after the last immunization was determined using a chromium release assay with HBsAg-expressing target cells, as previously described [18]. CTL activity was calculated as percentage of specific lysis at a given effector:target (E:T) ratio and expressed as group means of individual animal values, which were themselves the average of triplicate assays.

2.4. Statistical analysis

Data were analyzed using the GraphPAD InStat program (GraphPAD Software, San Diego). The statistical significance of the difference between group means was calculated with non-transformed data for percentage of specific lysis or with transformed data ($\log_{10}$) for ELISA titers by Student's 2-tailed t-test for two groups, or by 1-factor analysis of variance (ANOVA) followed by Tukey's test for three or more groups. Differences were considered to be not significant with $P > 0.05$.

3. Results

3.1. IN delivery of adjuvants and effect on plasma IgG

Delivery of HBsAg or TT (10 μg) without adjuvant by IN immunization induced no or only low levels of Ag-specific IgG in the plasma of mice, even after three immunizations (Fig. 1). In contrast, the addition of any

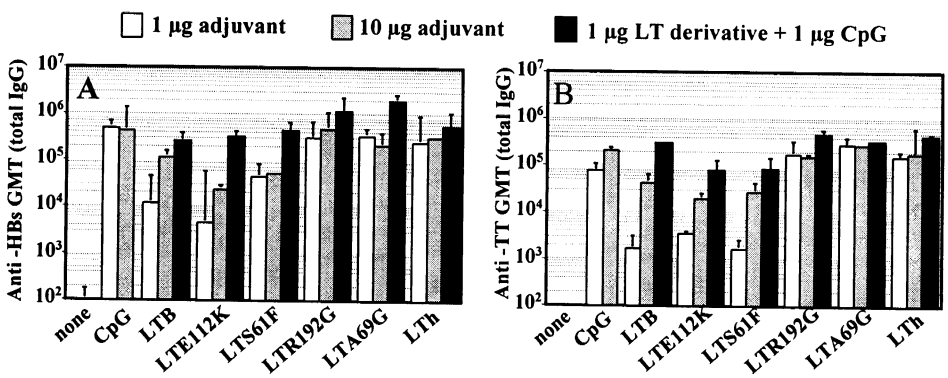

Fig. 1. BALB/c mice ($n = 5$) were immunized by IN delivery of 10 μg HBsAg (panel A) or TT (panel B) either alone (none) or with 1 μg (white bars) or 10 μg (gray bars) of CpG-containing ODN (CpG), LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh as adjuvant. In addition, other mice were immunized with CpG combined with one of the other adjuvants (1 μg each, black bars). Mice were boosted in an identical manner at 4 and 8 weeks. Each bar represents the group mean (± SEM) of the ELISA titer for HBsAg-specific (anti-HBs, total IgG) or TT-specific (anti-TT, total IgG) antibodies in plasma taken 4 weeks after third and final immunization.

single adjuvant significantly increased Ag-specific IgG levels ($P < 0.05$). With low dose adjuvant (1 μg), equivalent high IgG levels were induced by CpG ODN, LTh, LTR192G or LTA69G ($P = 0.87$ and 0.18 for HBsAg and TT respectively) and equivalent lower levels by LTB, LT112K or LTS61F ($P = 0.48$ and 0.68 for HBsAg and TT respectively). A higher adjuvant dose (10 μg) did not enhance Ag-specific IgG levels with CpG ODN, LTh, LTR192G or LTA69G ($P > 0.05$), and only did so with LTB, LT112K or LTS61F when TT was used as antigen ($P < 0.05$). TT-specific IgG levels induced with a low dose (1 μg) of LTR192G, LTA69G or LTh were higher than those obtained with a 10-fold higher dose (10 μg) of the other toxin derivatives ($P < 0.05$). With CpG ODN, LTh, LTR192G or LTA69G a single low dose immunization was sufficient to cause seroconversion in most or all mice, whereas with LTB, LT112K or LTS61F, a higher adjuvant dose or multiple immunizations were required (data not shown).

With IN delivery, the addition of CpG to any of the weaker bacterial toxin-derived adjuvants (LTB, LTE112K, LTS61F) (1 μg each), raised Ag-specific IgG titers in all cases compared to bacterial toxin-derived adjuvant alone, such that responses induced with 1 μg each of CpG + LTB, LTE112K and LTS61F were equivalent to those with 1 μg of the more enzymatically active adjuvants alone ($P > 0.05$) (Fig. 1). With HBsAg as antigen, CpG combined with LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh gave respective geometric mean titers 22-, 69-, 10-, 4-, 6- or 2-fold greater than LT derivative alone, but only with CpG combined with LTR192G and LTA69G were titers greater than CpG alone (2- and 4-fold respectively). With TT as antigen, CpG combined with LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh gave respective geometric mean titers 164-, 21-, 45-, 2-fold greater than, equivalent to, or 3-fold greater than LT derivative alone, and LTB, LTR192G, LTA69G and LTh all gave titers 3- to 5-fold greater than CpG alone. However, only when TT was used as antigen with CpG plus either LTh or LTB were the differences significant compared to CpG or LT derivative alone ($P < 0.05$) (Fig. 1). Nevertheless, the IgG2a/IgG1 ratio increased, suggesting the Type 1-like response of CpG ODN dominated over the much more Type 2-biased response with LT and the LT-derivatives (Table 1).

Table 1
Effect of adjuvant on IgG2a/ IgG1 bias of HBsAg- or TT-specific immune responses

Adjuvant[a]	Adjuvant dose (μg)	Anti-HBs[b]	Anti-TT[b]
		IgG2a/IgG1	IgG2a/IgG1
None	NA	–	–
CpG	1	1.20	0.4
CpG	10	2.00	2.27
LTB	1	0.13	0.01
LTB	10	0.03	0.01
LTB + CpG	1 each	0.17	1.33
LTE112K	1	0.03	0.01
LTE112K	10	<0.01	0.04
LTE112K + CpG	1 each	0.30	0.49
LTS61F	1	0.20	<0.01
LTS61F	10	<0.01	0.01
LTS61F + CpG	1 each	0.10	0.73
LTR192G	1	0.09	0.04
LTR192G	10	0.07	0.22
LTR192G + CpG	1 each	0.48	2.09
LTA69G	1	0.05	0.02
LTA69G	10	0.19	0.11
LTA69G + CpG	1 each	0.75	0.92
LTh	1	0.31	0.07
LTh	10	0.04	0.08
LTh + CpG	1 each	1.88	1.61

[a] BALB/c mice were immunized at 0, 4 and 8 weeks by IN immunization of 10 μg of HBsAg or TT without adjuvant (none) or in combination with CpG-containing oligonucleotides (CpG) and/or LTh, LTB, LTE112k, LTS61F, LTR192G, LTA69G as adjuvants.
[b] HBsAg- or TT-specific IgG1 and IgG2a antibodies were measured in plasma collected 4 weeks after final immunization from mice immunized using HBsAg or TT as antigen. The IgG2a to IgG1 ratios are reported, with a value >1 indicating a predominantly Th-1 like response.

3.2. IN delivery of adjuvants and effect on mucosal IgA

No IgA was detected in lung, gut, vaginal or saliva samples when HBsAg or TT was given IN without adjuvant. With a low dose of individual adjuvants, similar high levels of Ag-specific IgA were detected in mucosal samples with CpG ODN, LTR192G, LTH, or LTA69G ($P > 0.05$) IgA levels were lower with LTB, LTE112K, and LTS61F, and in general a higher adjuvant dose (10 μg) was required to attain titers greater than 100 (Fig. 2). Overall Ag-specific IgA titers were not significantly greater with adjuvant combinations than with the best of the single adjuvants.

3.3. IN delivery of adjuvants and effect on CTL

Only very low levels of CTL were induced after IN delivery of HBsAg alone or with a low dose (1 μg) of LTE112K, LTS61F, or LTB. Indeed even at a high dose, these adjuvants induced very low (LTS61F) or only moderate (LTB, LTE112K) levels of CTL. In contrast, equivalent high CTL activity was induced with CpG ODN, LTR192G, LTh or LTA69G, even when used at a low dose (Fig. 3). Combining CpG ODN with any of the bacterial toxins, did not further augment HBsAg-specific CTL activity (data not shown).

3.4. Oral delivery of adjuvants

Anti-TT IgG titers in the plasma of mice were significantly greater when TT was administered with any single adjuvant (10 μg CpG ODN, LTh, LTB, LTE112K or LTR192G) or CpG ODN/bacterial toxin adjuvant combination (1 μg each), than when TT was delivered alone ($P < 0.05$) (Fig. 4, left panel). Higher doses (10 μg) of all single adjuvants gave equivalent anti-TT IgG titers ($P = 0.35$). Lower doses of CpG ODN combined with LTh, LTB, LTE112K or LTR192G (1 μg each) gave equivalent responses as 10 μg of any adjuvant alone ($P > 0.05$), and there were no differences between different adjuvant combinations ($P = 0.24$).

Antibody isotypes were used as an indication of the Th-bias of the responses induced by oral administration with the different formulations. On its own, or with LT-derived adjuvants, TT gave a strong Type 2 response (IgG1 ≫ IgG2a). In contrast, with CpG ODN, the TT-specific response was mixed Type 1/Type 2, indicating that the CpG ODN shifted the response in a

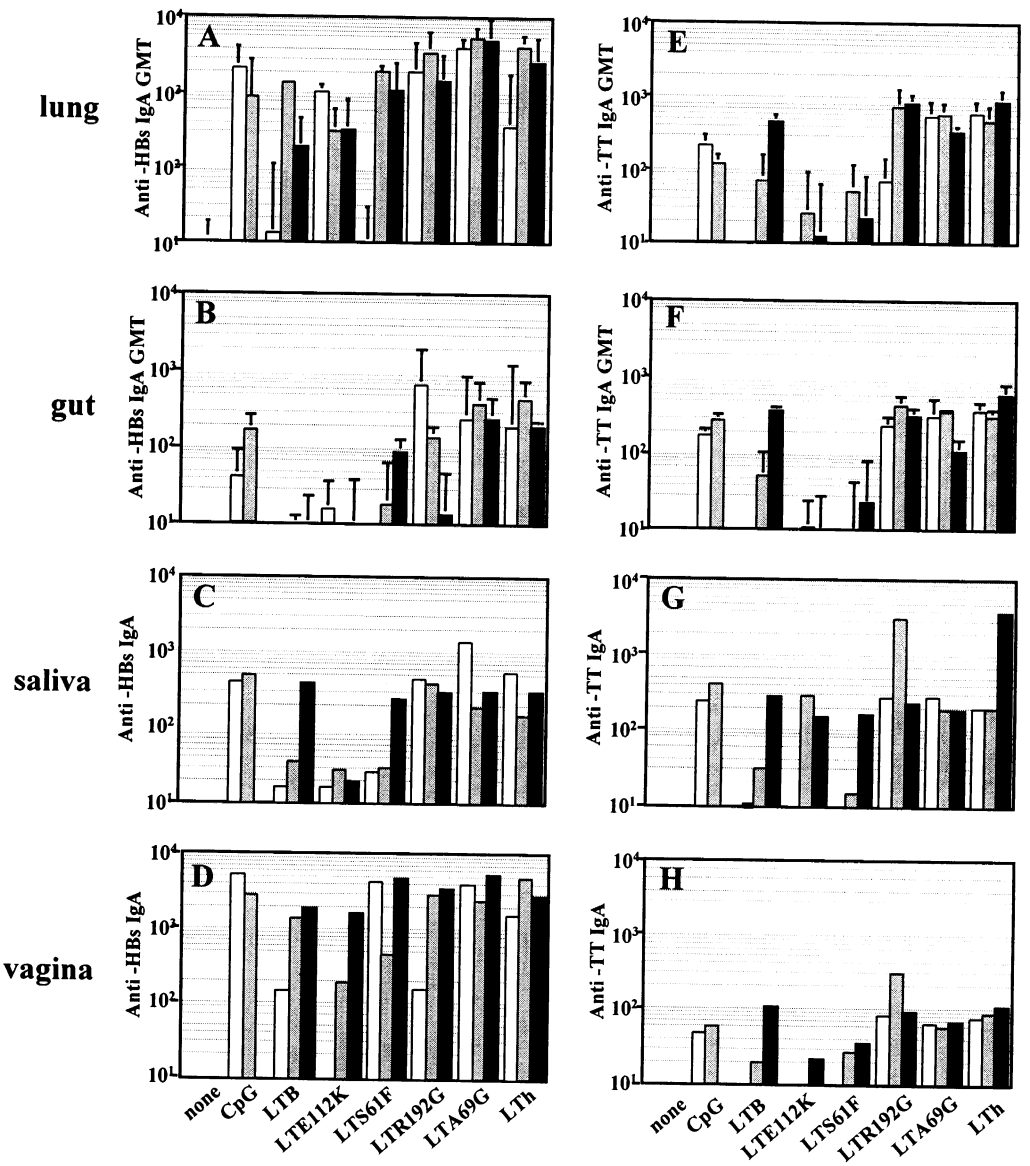

Fig. 2. BALB/c mice ($n = 5$) were immunized by IN delivery of 10 μg HBsAg (panels A–D) or TT (panels E–H) either alone (none) or with 1 μg (white bars) or 10 μg (gray bars) of CpG-containing ODN (CpG), LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh as adjuvant. In addition, other mice were immunized with CpG combined with one of the other adjuvants (1 μg each, black bars). Mice were boosted in an identical manner at 4 and 8 weeks. Panels A, B, E and F: Each bar represents the group mean (±SEM) of the ELISA titer for HBsAg-specific (anti-HBs IgA GMT) or TT-specific (anti-TT IgA GMT) antibodies in mucosal samples (lung, or gut washes) taken 4 weeks after third and final immunization. Panels C, D, G and H: Each bar represents the ELISA titer for HBsAg-specific (anti-HBs IgA) or TT-specific (anti-TT IgA) antibodies in pooled mucosal samples (vaginal wash or saliva) taken 4 weeks after third and final immunization.

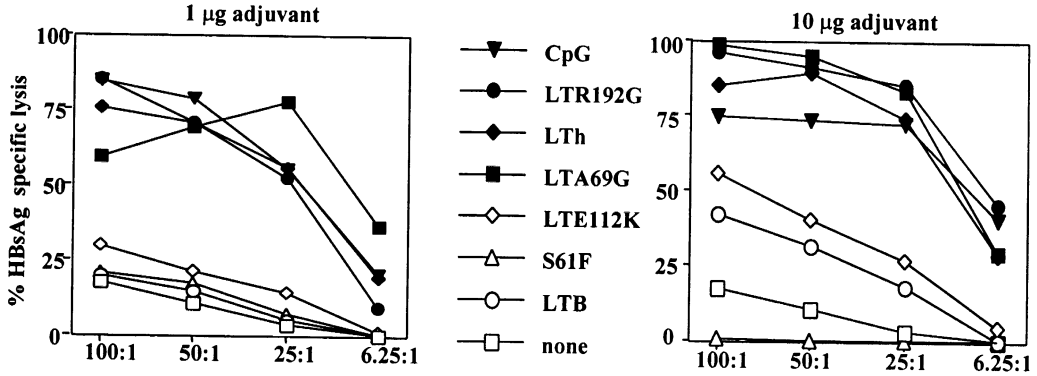

Fig. 3. BALB/c mice ($n = 5$) were immunized by IN delivery of 10 μg HBsAg either alone (none) or with 1 μg (left panel) or 10 μg (right panel) of CpG-containing ODN (CpG), LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh as adjuvant. Mice were boosted in an identical manner at 4 and 8 weeks. Spleens were removed 4 weeks after third and final immunization and CTL activity was determined. Horizontal axes indicate effector to target ratio (E:T) and vertical axes the HBsAg specific lysis as a percentage of the total possible lysis (% specific lysis).

Type 1 direction (Fig. 4, right panel). CpG ODN similarly enhanced Type 1-like responses when combined with any of the other adjuvants, even though the LT adjuvants on their own were strongly Type 2.

After oral administration of TT alone to mice, no TT-specific IgA was detected in mucosal samples (Fig. 5). In contrast, with all of the adjuvants it was possible to induce some level of IgA in at least some of the samples, although not all induced equivalent levels, nor did every adjuvant induce IgA in all types of mucosal samples. For example, CpG and LTR192G induced good levels of TT-specific IgA in lung, gut, saliva and vaginal washes of most mice, whereas LTE112K failed to induce IgA in saliva, gut or vaginal samples and only induced significant IgA in the lungs of 1 of 5 mice.

4. Discussion

The mucosal surfaces of the gastrointestinal, genitourinary and respiratory tracts are the primary sites of entry of most infectious agents, yet most current vaccines are delivered parenterally and as such induce little or no specific mucosal immunity. The development of mucosal vaccines for human use would be considerably hastened if safe and effective mucosal adjuvants could be found. Mucosal adjuvants such as LT and CT are highly effective in animal models but are generally considered to be too toxic for use in humans. Efforts to develop less toxic versions that would be safe for human use have focused on the use of the non-toxic B subunit that lacks enzymatic activity [1,2,24–27] or genetic alterations that would result in reduced enzymatic activity of the A subunit [2–6]. We had previously found that IN delivery of CpG ODN induced responses stronger than those with CTB and equivalent to those with CT [18,38]. Furthermore, a combination of CT and CpG ODN gave stronger responses than either alone, suggesting a synergistic effect. Since this effect was seen with CT and not CTB, we hypothesized that it was dependent on the enzymatic activity of the A subunit, and furthermore, that a similar synergy would be more evident with LT derivatives that retained a significant amount of cAMP activity than with those that did not.

The role of cAMP activity in synergy with CpG was assessed using three active site mutants (LTE112K, LTA69G, LTS61F), a LTR192G as well as native LT and LTB. These mutants have previously been shown to induce differing degrees of cAMP accumulation in cultured Caco-2 cells (LT > LTA69G > LTR192G > LTS61F = LTE112K), which correlated well with their ability to elicit production of antigen-specific Type 1 and Type 2 cytokines [6]. Herein, with IN administration of these LT derivatives on their own, we found that the different adjuvants all augmented Ag-specific immune responses, but they fell into two distinct groups for degree of adjuvant activity. Strong systemic (IgG and CTL) and mucosal (IgA) immune responses were obtained with LTh, the LTR192G and an active site mutant LTA69G. Weaker responses, especially CTL and IgA, were obtained with the other LT-derivatives tested, namely the LTB and the two active sites mutants, LTE112K and LTS61F. Thus, LT and derivatives with higher enzymatic activity (LTA69G, LTR192G) had the best adjuvant effect and those with little or no enzymatic activity (LTE112K, LTS61F) had a weaker adjuvant effect. Using the same LT derivatives with IN delivery, a previous study has shown all LT derivatives to give equivalent humoral responses [6]. It is possible that the differences in results in the two studies may have arisen due to differences in the immunization schedules (0, 1 and 2 weeks versus 0, 4 and 8 weeks) and/or dose of adjuvant (5 μg vs 1 or 10 μg).

Although some reports have shown LTB to be a very weak adjuvant [28,29,40], other studies have shown it to

be a fairly good mucosal adjuvant, especially for IN immunization [6,26,41], which is supported by our current findings. It has been suggested that differences between studies using LTB have arisen since, in some cases, the purified preparations of LTB used were subsequently found to contain some holotoxin contamination [28,29], however this cannot be true in all cases since recombinant LTB has been shown to be an effective mucosal adjuvant [26]. For an equivalent dose, IN administration of CpG ODN gave systemic and mucosal responses of similar strength to those of the stronger LT-group (i.e., LTh, LTR192G and LTA69G). This agrees with our earlier studies where we found similar adjuvant activities of CpG ODN and CT [18,19] or LT [38] with HBsAg as antigen. The equal CTL with CpG ODN and some LT-related compounds is interesting considering that antibody isotype suggested a more Type 1-like response with CpG ODN compared to the strongly Type 2 antibody response with LT and derivatives. This does not occur with IM immunization of adult mice, suggesting that it may be unique to mucosal delivery, however we have seen a similar such dissociation of Th-bias of antibody response and CTL activity with parenteral immunization of very young mice [42]. The mechanism for this is not clear. Nevertheless, the data indicate that antibody isotypes cannot in all cases be used to predict CTL activity.

We have previously demonstrated with IN delivery of HBsAg that a synergistic relationship existed between CpG ODN and the native toxins, CT and LT, but not with their non-toxic derivatives, namely CTB and LTK63 [18,38]. This suggested that the synergy might have been dependent upon the enzymatic activity of the native toxins. To further investigate this, herein we evaluated native LT, LTB as well as a number of different LT mutants (LTS61F, LTA69G, LTE112K, LTR192G) that have different degrees of remaining enzymatic activity. However, with HBsAg as antigen, we did not see any synergistic effect when CpG was combined with any of LT-derived adjuvants (i.e. LTh, LTS61F, LTA69G, LTE112K, LTR192G, or LTB), and with TT as antigen, only in mice immunized IN with CpG ODN plus LTh or LTB were the titers greater than those obtained with CpG ODN alone. Thus, these results suggest that using CpG ODN together with bacterial toxin derivatives will not induce higher antibody titers. CpG ODN and LT derivatives gave predominantly Type 1-like and Type 2-like responses respectively when used separately, but mixed responses (Type 1/Type 2) when combined. This contrasts somewhat with our earlier findings after IM injection, whereby responses obtained with a combination of CpG ODN and other adjuvants (alum, Freund's complete and incomplete adjuvants, monophosphoryl lipid A) were more Type 1-like than with any single adjuvant (including CpG ODN), even when the other adjuvant was strongly Type 2 [43]. Thus, while the Type 1-bias of CpG ODN was still able to exert its effect in the presence of a Type 2 stimulus, it was less dominant with mucosal than parenteral administration.

Following oral immunization, all adjuvants gave equally strong immune responses, including LTB. Thus, in this study, the gut-associated lymphoid tissue appears to be more susceptible to immune activation than the nasal-associated lymphoid tissue and/or bronchus-associated lymphoid tissue. Our findings are in contrast to those previously published using the same LT derivatives for oral delivery wherein ability to induce humoral responses correlated with ability to induce cAMP accumulation [6]. As with IN delivery, it is possible that the differences in adjuvant dose and immunization schedule may be responsible for differences in results between two studies.

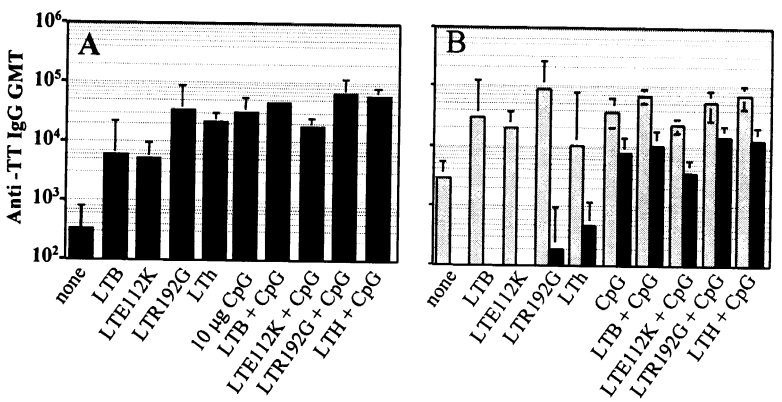

Fig. 4. BALB/c mice ($n = 5$) were immunized by oral delivery on days 0, 7, 14 with 10 μg TT either alone (none) or with 10 μg of CpG-containing ODN (CpG), LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh as adjuvant. In addition, other mice were immunized with CpG combined with one of the other adjuvants (1 μg each). *Panel A:* Each bar represents the group mean (± SEM) of the ELISA titer for TT-specific (anti-TT, total IgG) antibodies in plasma taken 1 week after third and final immunization. *Panel B:* Each bar represents the group geometric mean (± SEM) of the ELISA titer for TT-specific antibodies of IgG1 (gray bars) or IgG2a (black bars) isotypes in plasma taken 1 week after final immunization.

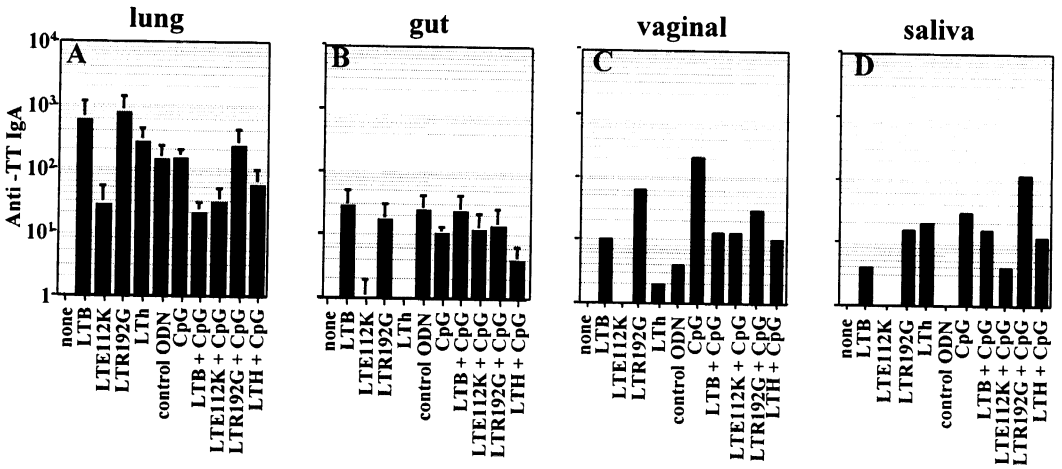

Fig. 5. BALB/c mice ($n = 5$) were immunized by oral delivery on days 0, 7, 14 with 10 μg TT either alone (none) or with 10 μg of CpG-containing ODN (CpG), LTB, LTE112K, LTS61F, LTR192G, LTA69G or LTh as adjuvant. In addition, other mice were immunized with CpG combined with one of the other adjuvants (1 μg each). Each point (panels A, B) represents the ELISA titer for Ag-specific IgA antibodies in lung and gut washes of individual animals, and bars (panels C, D) represent the ELISA titer for Ag-specific IgA antibodies in pooled vaginal washes and saliva.

In the current study, with both IN and oral routes of delivery, responses with CpG ODN were more Type 1-like than with any other adjuvant. A similar Type 1 bias of CpG ODN has previously been demonstrated after IN immunization using HBsAg [18,19], HBsAg/Ab complexes [44], whole killed influenza virus [21], and β-galactosidase [20]. Type 1-like responses in mice are generally associated with enhanced levels of IgG2a antibodies, which are thought to have superior neutralizing capabilities, and CTL, which are desirable for protection against numerous intracellular viral, bacterial and parasitic pathogens. In addition, Type 2 responses in the lung may be undesirable since they have been associated with the lung pathology of asthma [45,46]. The most commonly used mucosal adjuvant in animal models, CT, has recently been shown to induce inflammatory responses in the lung that are associated with increased IgE and IL-5 [47]. In contrast, a number of studies have demonstrated that IN administration of CpG ODN in mice can prevent allergen-induced asthmatic responses, including airway eosinophilia, Type 2 cytokine induction, IgE production and bronchial hyperactivity, by redirecting immune responses towards a more Type 1-like profile [48–53]. CpG motifs typically induce a Type 1-like pattern of cytokine production dominated by IL-12 and IFN-γ, with little secretion of Type 2 cytokines [31], and this likely accounts for their strong Type 1 immunomodulatory effects. Recent studies in a murine model of asthma have also demonstrated that CpG-ODN can down-regulate Type 2 responses in IFN-γ and IL-12 knockout mice, suggesting that CpG-ODN prevent the generation of Type 2-like immune responses by multiple mechanisms that involve, but do not require, IL-12 and IFN-γ [53].

The LT derivatives used in this study have previously been proposed as potential adjuvants for human vaccines to be delivered mucosally [6], and our findings support this. We have identified two LT mutants that both have strong adjuvant activity in mice, similar to that with LT. Further testing will be required to determine if this also holds true in larger animal models. Furthermore, toxicity testing will be required to determine whether they are likely to be safe and well tolerated in humans. Contrary to our initial hypothesis, we did not find any clear advantage in combining CpG ODN with bacterial toxin derivatives.

Acknowledgements

We are grateful to Amanda Boyd, Dawn Edmonds, Karen Wong, and Lu Zhang for excellent technical assistance. This work was supported by the Medical Research Council of Canada and Coley Pharmaceutical Group Inc. HLD is the recipient of a Career Scientist Award from the Ontario Ministry of Health.

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