

Production of IgE Antibody and Allergic Sensitization of Intestinal and Peripheral Tissues After Oral Immunization with Protein Ag and Cholera Toxin¹

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Cholera toxin (CTX) is a potent oral adjuvant for the induction of mucosal IgA Ab responses to protein Ags. We examined the Ab responses and allergic sensitization of several strains of mice to protein Ags, administered orally with CTX. The mice made strong IgA and IgG1 serum Ab responses, but little IgG2a Ab to Ags such as hen egg lysozyme (HEL) and OVA. However, when given a subsequent i.p. challenge with Ag alone, the same mice had immediate hypersensitivity reactions that included respiratory distress and death. Within 10 min of i.p. challenge, immunized mice had high levels of plasma histamine and extensive degranulation of mast cells in target tissues. These mice had detectable serum IgE Ab. Ag administered orally with the B subunit (CTB) of CTX did not sensitize mice. Intestinal tissues taken from these mice had Ag-specific ion-secretory responses in vitro, typical of intestinal anaphylaxis. Ag given s.c. without adjuvant could also sensitize for systemic and intestinal anaphylaxis. Sensitization with HEL given s.c. was dose dependent and correlated with a critical amount of HEL in the circulation. HEL was detected in the circulation after oral immunization, but CTX did not increase the uptake of HEL. Thus, oral immunization with a protein Ag in the presence of CTX can sensitize an animal for systemic and intestinal anaphylaxis. These results suggest a cautious approach to the use of CTX as an adjuvant in oral vaccines, and provide a new model to study immediate hypersensitivity reactions to intestinal Ag. *The Journal of Immunology*, 1994, 153: 647.

The adjuvant effect of cholera toxin (CTX)³ on the local immune response of the intestine has been studied extensively in animal models for many years (1). For mice, introduction of microgram to sub-microgram quantities of the whole holotoxin by gastric feeding or directly into the small intestine by injection results in large anti-CTX IgA Ab responses (2–4), and the magnitude of the response is related to the H-2 haplotype (4, 5). CTX can promote the intestinal IgA Ab response to protein Ags that are mixed with CTX, or covalently associated with CTX, during oral immunization (3, 4). At the same time, oral immunization with CTX can result in IgA

Ab secretion at distal mucosal tissues such as the salivary gland (6, 7). Studies in humans also indicate that CTX or its major sub-fragment, the B subunit (CTB), can promote mucosal IgA Ab production (8). These reports have encouraged research on the use of CTX or CTB in subunit vaccines for human use, with an emphasis on maintaining the adjuvant properties of CTX while eliminating its direct toxic effects (9).

Recent in vitro studies indicate that CTX can also have effects on the cytokine production by Th2 cells and on the isotype switching of B cells to IgE. CTX can stimulate macrophage in vitro to produce IL-1, a cytokine required as a costimulatory signal for Th2 cells (10–13). Epithelial cell lines can also produce IL-1 in response to CTX (14). CTX does not affect IL-4 production by Th2 type T helper cells, but significantly reduces IL-2 production by Th1 T cells in vitro (15). IgG1 and IgE Ab production by B cells is up-regulated in vitro by CTX in the presence of IL-4, showing that CTX could augment the activity of IL-4 during isotype switching to IgG1 and IgE by B cells (16, 17). CTB has a similar in vitro activity but is much less efficient (16). This direct effect on B cells is similar to the

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³ Abbreviations used in this paper: CTX, cholera toxin; HEL, hen egg lysozyme; pCytC, pigeon cytochrome c; SAP, streptavidin-alkaline phosphatase; Isc, short circuit current response.

effect of prostaglandins that increase cAMP and intracellular calcium (17).

CTX can promote IgG1 along with IgA Ab in vivo (18). Recent work by others suggested that Ag-specific IL-4-producing T cells are generated in intestinal lymphoid tissue soon after oral immunization with CTX (19, 20). Based on the ability of CTX to promote IgE Ab in vitro, one would predict CTX could act as an adjuvant for IgE production driven by IL-4, in vivo. To our knowledge, there has been no description of CTX stimulating IgE Ab production, in vivo. In this report, we describe a model of CTX induction of IgE Ab in vivo after oral immunization of mice with protein Ags including hen egg lysozyme (HEL) and OVA. Mice immunized orally with protein Ags and CTX were susceptible to systemic anaphylaxis when challenged parenterally with that Ag. In addition, their intestinal tissue exhibited an immediate hypersensitive response to Ag in an ex vivo physiologic assay. CTX did not increase the permeability of the intestine to Ags such as HEL. Thus, oral immunization in the presence of CTX can potentiate IgE Ab to protein Ag and thus produce a potentially life-threatening allergic state in the immunized animal.

Materials and Methods

Mice, Ags, and immunization protocols

Female C3H/He mice (Charles River Laboratories, Montreal, Canada), were purchased at 5 to 7 wk of age and housed in the central animal facility at McMaster Health Science Center. Female mice of other strains, BALB/c, DBA/1, B10.A, and SWR/J were purchased from The Jackson Laboratory (Bar Harbor, MN). Highly purified HEL, OVA, and pCytC were purchased from Sigma Chemical Co. (St. Louis, MO). For oral sensitization, six to eight mice were immunized orally with 200 to 1000 μ g of Ag mixed with 5 to 10 μ g of CTX (List Biologic Labs., Campbell, CA), in 300 to 400 μ L of 0.2 M sodium bicarbonate solution. In most experiments, the dose was 200 μ g for HEL and pCytC, and 1 mg for OVA. Some mice received CTB, the pentameric B subunit of CTX (List), to determine the requirement for the A chain of the holotoxin. The solution of CTX (or CTB) and Ag was administered intragastrically, by using a blunted needle. Mice received two identical doses, 3 wk apart. Some groups of mice were also given two s.c. immunizations (3 wk apart) with 30 μ g of Ag in PBS, or mixed with 5 μ g CTX in PBS, or emulsified in IFA. The s.c. injections were given in two 50 μ L doses, one to either hind haunch. The challenge i.p. injection consisted of 20 to 50 μ g of Ag per mouse in 200 μ L of PBS, and was given 3 wk after the second immunization (oral or s.c.). For analysis of circulating HEL, mice were given s.c. injections (two 50 μ L doses) of various amounts of HEL in PBS, into both hind haunches, or oral dosages as described above.

ELISA assays

Anti-HEL Ab titers in serum were determined by ELISA with the use of SAP: streptavidin-alkaline phosphatase (Sigma Chemical Co.) and biotin-conjugated anti-mouse IgA and IgG subclass Abs (Southern Biotechnology Assoc., Birmingham, AL). These assays measured end-point titers, based on the dilution of serum producing a signal of two times background (O.D. in the absence of serum). Mice were bled from the retro-orbital plexus, and sera was isolated after 1 h of clotting at 23°C. Typically, 100 μ L of Ag (5–10 μ g/ml) in borate-buffered saline (0.05 M borate, 0.15 M NaCl, pH 8.6) was incubated in microtiter plates wells for 3 h at 23°C. For IgA assays we used Maxisorb (NUNC, Denmark) plates for greater sensitivity. Plates were blocked with 10 mg/ml BSA in TBS (0.02 M Tris, 0.15 M NaCl, pH 7.2), then dilute sera (in 1 mg/ml BSA in TBS), were incubated for 16 h at 4°C. For IgG subclass analysis, the starting dilution was 1/50 and for IgA Ab it was 1/20. After serum

incubation, the plates were washed and then dilute (in BSA-TBS) biotin-anti-Ig Abs were added for 2 h at 23°C. This was followed by washing, SAP incubation, and finally *p*-nitrophenyl phosphate disodium substrate (Sigma Chemical Co.). Developed color was read by using a 405 nm filter. The IgG2a and IgG1 subclass reagents were tested for relative sensitivity by analysis of binding to various amounts of six different IgG2a and IgG1 mAb in an ELISA. Each of the reagents recognized the appropriate subclass at a sensitivity of approximately 5 ng (range 2–10 ng for both). Thus, we could compare the results of the two assays for relative amounts of IgG2a and IgG1 Ab.

For the IgE Ab assay, the rat anti-IgE mAb, LO-ME-3, was obtained as ascites (University of Louvain, Belgium), purified, and labeled with NH-biotin (Sigma Chemical Co.) by standard protocol (21). This mAb reacted with myeloma IgE proteins, but not IgA, IgG1, IgG2a, or IgG2b myeloma proteins in a solid phase ELISA test (not shown). Maxisorb plates were coated with 50 μ L of 10 μ g/ml HEL, and procedures were followed as above, with serum dilutions beginning at 1/5 and by using the biotin-LO-ME-3 to detect bound IgE Ab. After SAP incubation, sensitive detection of bound enzyme was performed by using Immunosellect reagents (GIBCO BRL, Gaithersburg, MD) and 492 nm filter. The Immunosellect reagent is highly sensitive because it relies on the alkaline phosphatase (bound to Ab positive wells) to cleave a phosphate group from NADPH, yielding an NADH product that in turn catalyzes the reduction of idonitrotetrazolium dye (in the presence of diaphorase and alcohol dehydrogenase) to the colored formazan product. The high sensitivity of the assay is thus the result of a double enzyme amplification.

Circulating quantities of HEL were detected in the sera of mice given oral or s.c. injections by using a quantitative sandwich ELISA. For this, 50 μ L of anti-HEL mAb HYHEL-8 (22) (purified from ascites in our laboratory) at 1 to 4 μ g/ml in borate-buffered saline was allowed to bind to Maxisorb microtiter wells. The plates were blocked with BSA, and then test serum samples or HEL in dilute (1/10) normal mouse serum were added and incubated for 1 h at 37°C. The plates were washed and then 50 μ L of 100 ng/ml of biotin-conjugated HYHEL-5 (22) (purified from ascites and labeled in our laboratory) was added, for an incubation of 1 h at 37°C. This was followed by SAP, and then Immunosellect reagents, as above. Preliminary studies of HEL concentration in serum after s.c. injection indicated maximum concentrations at 10 to 20 min, with one-half of the maximal concentrations by 3 h. This assay could detect concentrations of HEL as low as 1 ng/ml. HYHEL-8 and HYHEL-5 recognize independent conformational determinants on HEL (22). Therefore, this assay could detect native HEL in serum. The hybridoma cell lines for HYHEL-8 and HYHEL-5 were the generous gift of Dr. S. Smith-Gill, National Institutes of Health, Bethesda, MD.

Histochemical analysis of mast cell degranulation

A 0.5-cm segment of tongue was removed from mice, immediately after death by cervical dislocation, and fixed in freshly prepared Carnoy's fixative (60% ethanol, 20% chloroform, and acetic acid) for 18 h at 23°C. Individual tissues were taken and coded, to eliminate observer and sampling bias. The fixed tissues were processed and paraffin embedded, according to routine procedures. Sections of tissue (5–7 μ M) were cut and stained to detect mast cells by using toluidine blue, at pH 0.5, for 16 to 18 h. After staining and mounting, sections were viewed by light microscopy at $\times 200$ magnification to count the total number of mast cells. Cells were counted when the stained cell was a complete cross-section or when the nucleus was visible. The extremities of the tissue were not examined in any section, because mast cells in those areas were frequently disrupted by tissue handling. Instead, mast cells were counted in the musculature and along the deep blood vessels. A degranulated mast cell was defined as a toluidine-positive cell with five or more distinct stained granules (identified at $\times 400$ magnification) completely outside of the cell. Usually three to four sections were counted (50–300 cells per section) for each tissue specimen.

Histamine assay

A radio-enzymatic assay for plasma histamine was performed as previously described (23). Mice were bled from the retro-orbital plexus under ether, 1 to 2 h before i.p. challenge with HEL, and similarly after i.p. challenge. An initial experiment indicated that the histamine levels in responding mice bled at 5 to 10 min. post challenge was not different than that in mice bled at 10 to 20 min. Subsequent experiments always used

plasma collected at 8 to 10 min. post challenge, for both immunized and control groups. It was necessary to obtain blood by cardiac puncture from some mice with severe reaction to Ag challenge, because sufficient blood could not be drawn from the orbital plexus. In those experiments, an equal number of control group mice were given cardiac bleeds, to control for any effects on plasma histamine caused by cardiac bleeding. Bleeds from individual mice were taken and coded to avoid sampling bias in the subsequent assay. Approximately 200 to 300 μ l of fresh blood was mixed with 30 μ l of 0.5 M EDTA, and immediately centrifuged. 100 μ l of the resulting plasma was diluted 1/4 in 0.2 M phosphate buffer, pH 7.6, suitable for the radio-enzymatic assay. The diluted plasma samples were placed in boiling water for 5 min. (to inactivate endogenous histaminase), centrifuged at 10,000 rpm, and then frozen at -20°C until assay. The assay has a detection limit of approximately 1 ng histamine in a 100 μ l sample.

Measurement of intestinal anaphylaxis

The response of intestinal tissues to Ag challenge *ex vivo* was evaluated in Ussing chambers, by recording changes in short circuit current response (Isc), as previously described (24). Briefly, 1- to 2-cm segments of freshly isolated whole jejunal tissue were mounted in Ussing chambers and incubated in oxygenated Krebs buffer at 37°C . After equilibration of tissue and measurement of baseline conductance and Isc, HEL was added to either the serosal side or luminal (epithelial) side of the tissue, at a concentration of 100 $\mu\text{g}/\text{ml}$. Isc was continuously monitored over the next 15 min. This technique measured the net charge transfer (essentially the chloride ion secretion) from the tissue in response to Ag. Previous work had indicated that the response was inhibitable by antihistamines and mast cell inhibitors (24–26).

Statistical analysis of histamine, mast cell degranulation, and intestinal secretory data

The data for histamine response mast cell degranulation and intestinal secretory response were analyzed by one-way ANOVA, with a "Tukey" comparative parametric analysis of different groups. This was done because control groups were largely nonresponsive (zero) or had very small variance relative to treated groups, rendering standard parametric analysis (such as Student's *t*-test) invalid. For the ANOVA, confidence was set at 95%, so that resulting "F-values" that were greater than 1.0 indicated a large probability that differences exist between the groups. The Tukey analysis indicated which groups were significantly different from one another, usually at a confidence of greater than 98%.

Results

Oral immunization with HEL and CTX primes mice for an immediate systemic reaction, after i.p. challenge with HEL

Four strains of mice were orally immunized twice with a mixture of HEL and CTX, with a 3 wk interval between (see *Materials and Methods*). These mice were then challenged 3 wk later with HEL by i.p. injection. Many mice died within 1 h of challenge, in three of the four strains of mice examined (Table I). A distinct reaction pattern and behavioral change was observed in the mice within 10 to 20 min of challenge, including scratching and rubbing about the nose and head, standing still with change in respiratory rate, puffiness around the eyes, and loss of consciousness. Mice immunized orally with HEL alone or left untreated did not have this reaction after i.p. challenge with HEL.

To determine whether we had induced an Ag-specific anaphylactic response in those mice, we immunized a separate group of C3H mice and examined their plasma for increased histamine content and their tongue tissue for

Table I. Different strains of mice immunized orally with HEL and CTX have fatal response to i.p. challenge with HEL

Strain	H-2	Oral Immunization ^a		Death/Total ^b
		HEL	CTX	
C3H/He	k	+	+	5/8
	k	+	–	0/4
B10.A	a	+	+	0/8
	a	+	–	0/4
SWR/J	q	+	+	5/7
	q	+	–	0/5
DBA/1	q	+	+	3/5
	q	+	–	0/4

^a Mice were given HEL $\pm$ 10 μg CTX orally twice, 3 wk apart. For mice given CTX: four of eight C3H and B10.A, four of seven SWR/J mice, and three of five DBA/1 mice received 1 mg with CTX. The remainder of mice given CTX were also given 0.1 mg of HEL. All mice not given CTX received 1 mg of HEL, twice.

^b Death was observed within 1 h for most strains.

mast cell degranulation, after i.p. challenge. The mice were bled before i.p. challenge with HEL and 5 to 20 min after i.p. challenge, to obtain plasma. At the same time, tongue tissue samples were obtained and fixed, for later analysis of tissue mast cells (see *Materials and Methods*). The majority (8 of 10) of the mice immunized orally with HEL and CTX had large amounts of plasma histamine after i.p. challenge (Fig. 1A). Only one of five mice, immunized with HEL alone, responded with a small increase of plasma histamine. The difference in histamine response between the mice immunized with CTX and HEL and the control groups was statistically significant ($F = 13$, $p < 0.001$) and the control groups were not different from each other (95% confidence). In addition, eight of nine mice immunized with CTX and HEL had substantial mast cell degranulation, whereas those immunized with HEL alone had essentially normal mast cells (Fig. 1B). This difference was statistically significant ($F = 16$, $p < 0.001$). The total numbers of mast cells per section were similar for the three groups of animals. Sections from animals given CTX and HEL had between 52 and 211 mast cells, those from HEL immunized mice had between 100 and 315 mast cells, and those from untreated mice had between 75 and 206 mast cells. Figure 2 shows a striking correlation of histamine and mast cell response for individual mice immunized with CTX and HEL, consistent with mast cell-mediated histamine release. These data indicated that oral immunization with the use of CTX had sensitized the C3H mice for a systemic anaphylactic response to HEL, upon parenteral challenge.

IgE and IgG subclass Ab response in orally immunized mice

We measured anti-HEL Ab of the IgA, IgG1, and IgG2a isotypes in the serum of orally immunized mice, by using standard isotype-specific ELISA assays. A sensitive ELISA assay that detected IgE Ab was developed (see

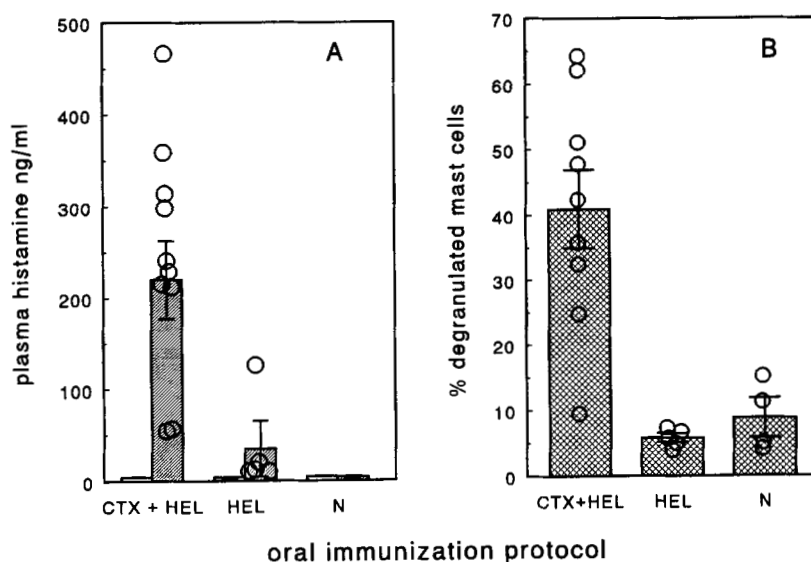


FIGURE 1. Systemic histamine response and mast cell degranulation by orally immunized mice when challenged i.p. with Ag. A. Mean ($\pm$ SEM) plasma histamine concentrations from mice orally immunized with 200 μ g HEL and 10 μ g CTX (CTX + HEL), 200 μ g HEL alone (HEL), or not immunized (N), before (open bar) or 5 to 15 min after (hatched bar) i.p. challenge. Individual values ($\circ$) are shown for post-challenge plasmas. B. Tongue mast cell degranulation as a percentage of total mast cell count. Tissues were taken at the time of post-challenge bleed of mice in A. Hatched bars are mean values ($\pm$ SEM), and individual values ($\circ$) are shown. The number of mast cells counted per total area of section did not vary greatly between these groups (see text).

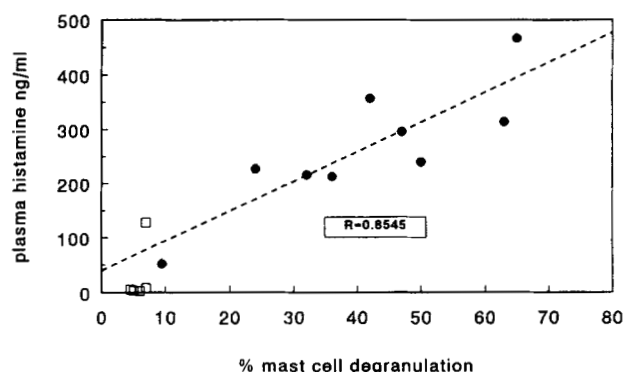


FIGURE 2. Correlation of histamine response and mast cell degranulation. Same data as in Figure 1 but expressed as a correlate of plasma histamine and mast cell degranulation for individual mice from the CTX plus HEL group ($\bullet$) or the HEL only group ($\square$). The regression R value is shown.

Materials and Methods) and used to detect anti-HEL IgE. Mice immunized orally with HEL and CTX showed IgG1, IgG2a, IgA, and IgE responses to HEL (Fig. 3). In contrast, those immunized with HEL alone had no detectable Ab when compared with nonimmunized controls. Because the titers on pooled sera indicated larger quantities of IgG1 Ab when compared with IgG2a, we analyzed individual sera from a separate experiment to determine whether this held true. Mice immunized with CTX and HEL had a

mean serum titer of IgG1 Ab that was 4.5 times greater than that of IgG2a Ab (Table II). A ratio of IgG1 to IgG2a Ab titers for each mouse was calculated and the mean value was 9.0. Similar data were obtained when we analyzed IgG1 to IgG2a ratios in the sera from other strains of mice, such as DBA/1 or SWR/J (not shown). The coincident observation of IgE Ab and large IgG1 to IgG2a ratio are indirect indicators of a strong Th2 response by these mice. Oral immunization with CTX and HEL also resulted in IgA Ab response, whereas HEL alone did not (Table II).

Oral immunization requires CTX holotoxin to generate anaphylactic sensitivity

C3H mice were sensitized orally with HEL and CTX or with HEL and a similar quantity of the pentameric B subunit (CTB), to determine whether the holotoxin was required for sensitization. The histamine response of mice immunized with HEL and CTB was not significantly different than that of control nonimmunized mice and mice immunized with HEL and CTX had a typical large histamine response (Fig. 4). Thus, CTB was not able to potentiate the anaphylactic sensitivity that the holotoxin could. The mice given CTX and HEL made large amounts of IgG1 and IgA anti-HEL Ab, whereas mice immunized with CTB and HEL had no significant IgG1 or IgA Ab response (not shown).

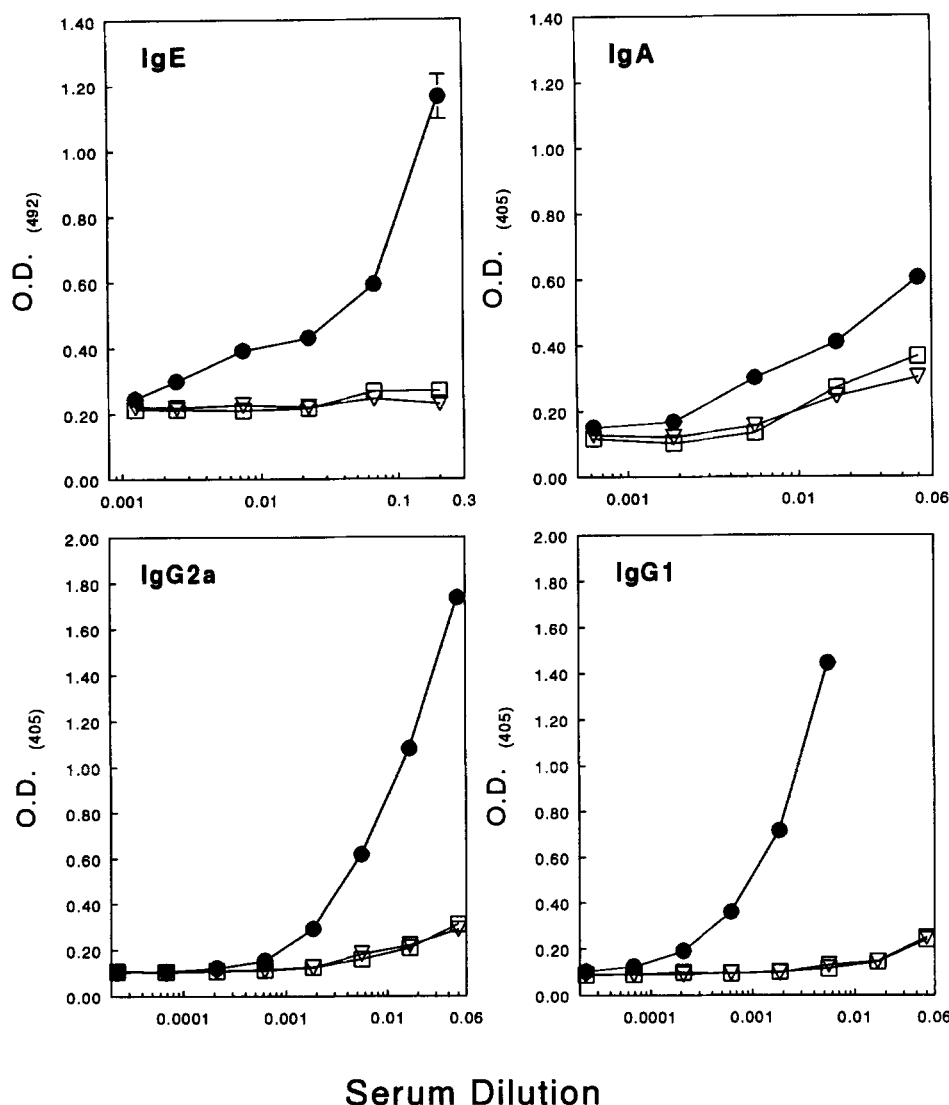


FIGURE 3. Ab response of mice immunized orally with HEL and CTX. Pooled sera from mice orally immunized with 200 µg HEL and 10 µg CTX (●), 200 µg HEL alone (□), or not immunized (▽), were assayed for IgE, IgA, IgG1, or IgG2a Ab against HEL in ELISA assays. The same pooled sera were used for each Ig isotype assay. Values are average OD from duplicate wells with range (error bars).

Comparison of s.c. and oral challenge for systemic sensitization

Although oral CTX was required for sensitization of mice for systemic anaphylaxis to HEL challenge and for production of IgE Ab, it was important to determine whether this sensitization could be achieved with parenteral immunization. Groups of mice were immunized s.c. with HEL alone, HEL with CTX, or HEL with IFA. Their IgE Ab responses and their histamine responses to i.p. challenge with HEL were compared with those of mice immunized with HEL and CTX by the oral route or those of control mice. All groups of mice given s.c. immunization responded with histamine release after challenge, as did five of eight mice in the orally immunized group (Fig. 5). The difference between

these groups of mice and the control mice was statistically significant ($F = 7, p < 0.01$). All three groups of s.c. immunized mice and the orally immunized mice produced anti-HEL IgE Ab (Fig. 6). Thus, s.c. injection of HEL was sufficient to induce an IgE Ab response by C3H mice and to sensitize the mice for systemic anaphylaxis. At this dosage of HEL s.c., the addition of CTX had little effect on either IgE production or histamine release.

CTX potentiates hypersensitivity to some but not all protein Ags during oral immunization

We tested both OVA and pCytC as oral Ags with CTX. OVA could sensitize either BALB/c or C3H mice when

Table II. IgG1, IgG2a, and IgA serum anti-HEL Ab in orally immunized C3H mice

Treatment	Mean Titer (SEM) ^a			G1/G2a Ratio ^b
	IgG1	IgG2a	IgA	
HEL + CTX	843 (243)	181 (80)	242 (118)	9.0
HEL	<50 ^c	<50	<20	
no treatment	<50	<50	<20	

^a Groups of six to eight mice were orally immunized as described in Figure 3 legend (two separate experiments), and end-point Ab titers were measured by ELISA in individual sera as described in *Materials and Methods*.

^b Ratio of IgG1 to IgG2a serum Ab titers from individual mice were calculated. The number shown is the mean ratio for the group.

^c < indicates limit of detection ie. minimal dilution of 1/50 or 1/20.

given orally with CTX or when given s.c. (Fig. 7). The IgG Ab response of the BALB/c and C3H mice was predominantly IgG1 in subclass and IgA Ab was also produced (not shown). Thus, mice could be sensitized to a second commonly used Ag given orally with CTX. In contrast, comparable (molar) dosages of pCytC given orally with CTX did not sensitize either BALB/c or C3H mice for significant histamine response (not shown). Thus, not every protein Ag given under a similar protocol will sensitize mice in the presence of CTX.

Intestinal anaphylaxis in both orally and s.c. immunized mice

We compared the response of intestinal tissues from mice sensitized to HEL by oral immunization or s.c. immunization protocols. We made use of an ex vivo system that measures the ion-secretory response of the tissue when challenged with Ag, in which increased *Isc* indicates increased secretory response (see *Materials and Methods*). Intestinal tissues taken from mice immunized orally with CTX and HEL or s.c. with HEL alone and challenged with HEL responded with large increases in *Isc* (Fig. 8A). Those tissues responded when either the serosal or luminal (epithelial) surfaces of those intestinal tissues were challenged with HEL. Tissues from mice immunized orally with HEL alone, with CTX alone, or from unimmunized mice did not respond at all to HEL challenge. Differences between treated and control groups were statistically significant ($F = 9$, $p < 0.001$, confidence = 95%). There were no significant differences in the response of serosal or luminal tissues when compared between the HEL-s.c. and HEL-CTX groups. Tissues from mice immunized with CTX and HEL did not respond to irrelevant Ags such as OVA (not shown). Figure 8B shows typical tracings of *Isc* responses by tissues from different mice, illustrating the magnitude of the response relative to the baseline. Thus, both oral immunization with HEL and CTX and s.c. immunization with HEL alone sensitized mice for intestinal anaphylaxis.

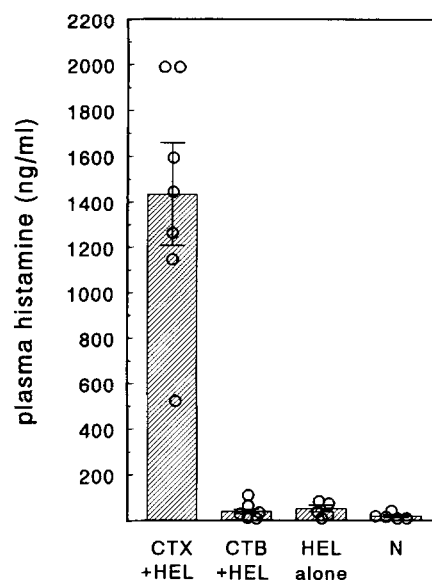


FIGURE 4. CTB does not potentiate allergic sensitivity to orally administered Ag. C3H mice were immunized and challenged as described in *Materials and Methods* and in Figure 1A. Some mice received CTB in molar equivalent to CTX. Plasma histamine concentrations after i.p. challenge are shown as described in Figure 1A.

HEL serum concentrations after s.c. immunization predicts a hypersensitive state

Because s.c. injection of HEL could induce a hypersensitive state in peripheral tissues, it was possible that a threshold level of HEL in circulation, after the primary immunization, might predict sensitization of the mice. This seems to be the case. Data in Figure 9 indicate that mice given less than 3 μ g of HEL s.c. failed to generate detectable histamine response with a subsequent challenge. Mice given an injection of 1 μ g of HEL s.c. had on average 68 ng/ml of HEL in circulation 15 min after injection, when serum concentrations are maximum (see *Materials and Methods*). Only three of six mice given 3 μ g of HEL were sensitized for a histamine response. The apparent responding groups (HEL = 3, 10, or 30 μ g) were significantly different ($F = 17$, $p < 0.001$) from the nonresponse groups (N, and HEL = 1 μ g). Responding groups were not significantly different from each other and neither were the control groups (N and HEL oral). A correlate of histamine response and circulating HEL (not shown) indicated that serum concentrations of native HEL that exceeded 200 to 300 ng/ml predicted parenteral sensitization for a subsequent challenge.

CTX does not increase the access of oral HEL to peripheral circulation

The question then arose whether the adjuvant effect of CTX on oral immunization with HEL was related to increased uptake of HEL to the circulation. In several experiments, mice were given HEL orally with or without

FIGURE 5. HEL sensitizes mice orally with CTX or s.c. without CTX or other adjuvants. Histamine concentrations are shown as in Figure 1A, with pre-challenge (open bars $\pm$ SEM) and 10 min post-challenge (hatched bars $\pm$ SEM). Groups of mice were immunized orally with 10 μ g CTX plus 200 μ g HEL (CTX + HEL oral), 200 μ g HEL alone (HEL oral), or were immunized s.c. with 30 μ g HEL alone (HEL s.c.), 10 μ g CTX plus 30 μ g HEL (CTX + HEL s.c.), or 30 μ g HEL in IFA (IFA + HEL s.c.). Individual values ($\circ$) are shown for post-challenge plasmas.

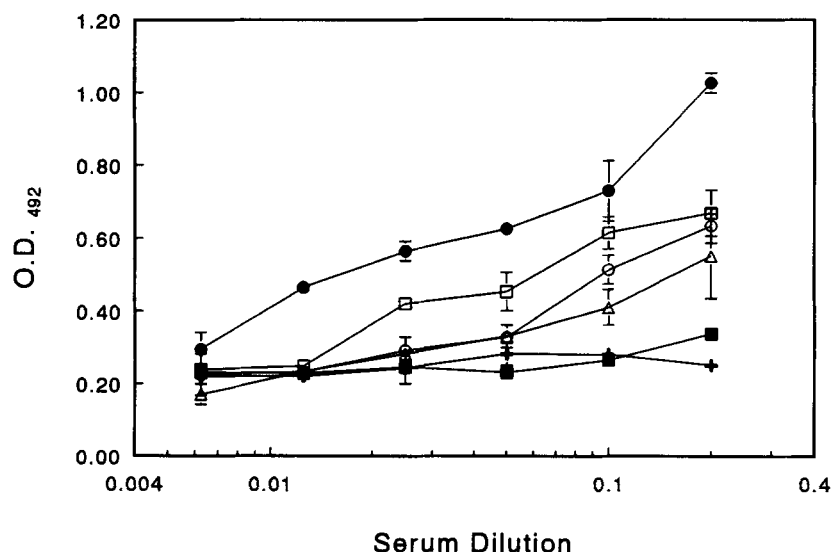
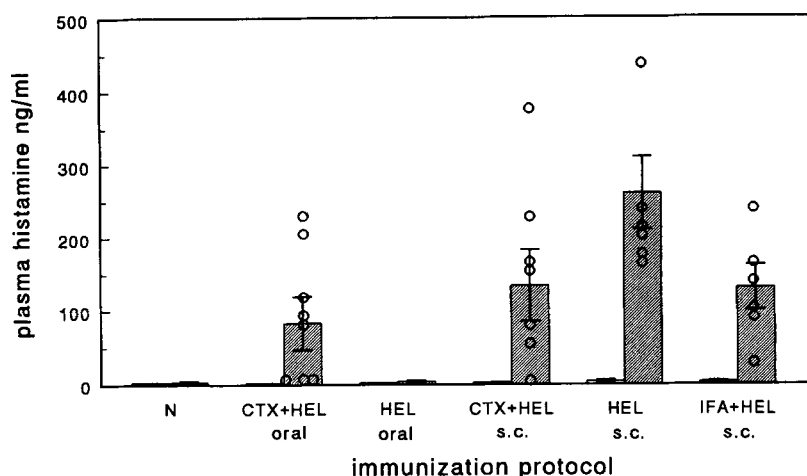


FIGURE 6. IgE Ab produced after immunization orally with CTX and HEL or s.c. with HEL. Titration of IgE Ab in pooled sera from the same groups of mice immunized as described in Figure 4 legend. These were groups immunized orally with CTX plus HEL ($\bullet$), or HEL alone ($\blacksquare$), or immunized s.c. with HEL alone ($\square$), CTX plus HEL ($\circ$), HEL in IFA ($\triangle$), or not immunized (+). Values are average O.D. from duplicate wells with range (error bars).

CTX. Their sera were examined for the presence of intact HEL, between 0.5 and 6 h thereafter. HEL was detected in the sera of mice given HEL orally and in those that were given HEL mixed with CTX (Table III). HEL was detected as early as 1 h after oral inoculation and began to decline between 4 and 6 h (not shown). There was no difference in the number of mice with detectable HEL in serum, nor in the mean serum concentration of HEL, between the two groups of mice. No mice had serum HEL at concentrations greater than 200 ng/ml at any point after oral immunization, with the standard dose of 200 μ g HEL. Higher serum concentrations of HEL were found in mice given larger doses (1 mg), but co-administration of CTX had no effect (Table III, Expt. 1).

Discussion

Our studies indicate that protein Ags, such as HEL or OVA, when given orally with CTX, can induce a hyper-

sensitive state in mice, such that a subsequent parenteral challenge with that Ag leads to a classical anaphylactic response. We have characterized this response as including a rapid and extensive degranulation of tissue mast cells and a rapid spike in plasma histamine. In addition, the mice showed signs of respiratory distress, edema, and in some cases died. In contrast, mice immunized orally with similar amounts of HEL or OVA alone exhibited none of these responses or symptoms when challenged parenterally with Ag. Ag given with CTB rather than CTX did not result in sensitization, indicating that the B subunit was insufficient and that the whole toxin including the toxic A chain was required.

Our data indicate that many but not all strains of mice are sensitized when immunized orally with a specific protein Ag in the presence of CTX. Some of the strains (C3H, B10.A) are known to be genetic high responders to HEL (27). These mice express the I-A^k molecule to which an

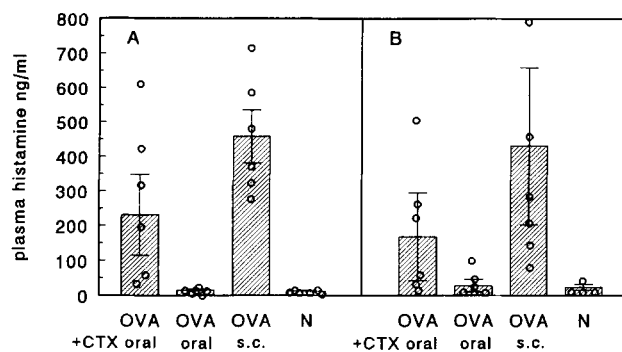


FIGURE 7. A second Ag, OVA, can sensitize two different strains of mice when given orally with CTX. BALB/c (A) and C3H (B) mice were immunized orally with 1 mg OVA plus CTX, OVA alone, or immunized s.c. with 30 μ g of OVA, by using procedures otherwise identical to previous experiments for HEL immunization. The mice were challenged i.p. with 30 μ g of OVA. Hatched bars are mean post-challenge histamine ($\pm$ SEM). Individual values are shown ($\circ$).

immunodominant peptide of HEL binds with high affinity (28, 29). However, we have observed that B10.A mice do not develop this hypersensitive state (Table I), even though they also express the I-A^k molecule. Lack of response by C3H mice when immunized with pCytC plus CTX illustrates again that the presence of the appropriate restriction element (I-E^k) for an immunodominant peptide (AA 88–104) is not sufficient for oral sensitization in the presence of CTX (30). The differences may lie in regulation through non-MHC gene products (31) or in the relative susceptibility of different proteins to degradation in the murine intestine. It should also be noted that C3H (H-2^k) and BALB/c (H-2^d) are low responder strains to CTX itself, whereas SWR/J and DBA/1 mice (H-2^q) are high responders (4, 5). Yet, all four strains can be sensitized in the presence of CTX. Our results are in keeping with the observation that response to protein Ags in the presence of CTX is independent of strain response to CTX and is related to other factors that control strain response to particular Ags (4).

C3H mice immunized orally with HEL and CTX produced HEL-specific, IgE Ab. The same is true when C3H mice were immunized parenterally (s.c.) with HEL alone. This latter observation is in accord with those of Bernstein et al. (32), who found that soluble protein Ags can induce IgE Ab when given in appropriate dosages, parenterally. The presence of IgE Ab is consistent with a model of IgE-mediated systemic anaphylaxis, although our data do not rule out a role for anti-HEL IgG Ab in triggering mast cell degranulation and histamine release (33). The production of IgE in response to oral immunization with HEL and CTX was accompanied by both IgG1 and IgG2a Ab, with IgG1 titers substantially higher than IgG2a titers. Similar high IgG1 responses were seen in mice immunized with OVA plus CTX. The association of high IgG1 response

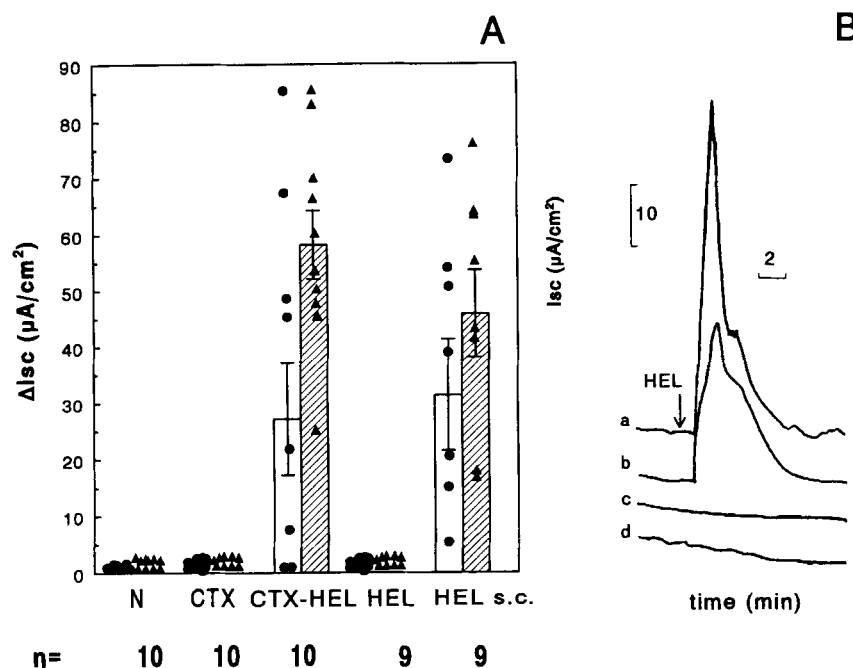
with IgE response is indicative of B cell responses driven by IL-4 production and a T helper response biased to Th2 cell activity (34–37). There is now good evidence that IL-4-producing T cells develop in intestinal lymphoid tissue after oral immunization in the presence of CTX (19, 20, 38), but the effects of intestinally derived Th2 subsets on IgE Ab production have not been examined in detail. High IgG1 and IgE Ab titers would also be consistent with the direct effects of CTX on B cells in isotype switching to IgG1 and IgE, especially in the presence of IL-4 (16). It remains to be seen whether HEL-specific B cells producing IgE Ab differentiate in the intestinal environment or in peripheral tissues after oral immunization with CTX and HEL. This may be an important consideration in light of the recent work of Auci et al., who argue that IgE-producing B cells may originate in the Peyer's patch of mice, even when mice are immunized parenterally (39).

Oral immunization with HEL and CTX or s.c. immunization with HEL alone both resulted in sensitization of intestinal tissue for anaphylactic response to Ag, in an ex vivo challenge. Previous work in the rat and mouse has indicated that the immediate type ion-secretory response of intestinal tissue is related to intestinal mast cells and IgE Ab (24, 25). Our data is consistent with a model of IgE-mediated intestinal anaphylaxis.

We did not observe an increase in the uptake of orally administered HEL (a 14,700 m.w. protein) into circulation in the presence of CTX, in contrast to a recent report by Lycke et al. (40). Their studies showed the direct measure of the uptake of 3,000 m.w. dextran, but they did not report the direct measure of the uptake of KLH, a protein Ag that when given orally with CTX induced a large Ab response in mice. Many other studies indicate the adjuvant effect of CTX on the intestinal response to various protein Ags, but uptake of Ag was not studied (3, 4, 7, 41). It may be presumed that as the majority of the Ab response is to conformational determinants on a native protein Ag, such as HEL (22, 42), at least some intact Ag can be taken up and made available to Ag-specific B cells. Our data argue that uptake of intact protein Ags to the circulation does occur (especially at large doses) but it is not augmented by CTX. Because CTX stimulates Ab production when given orally with 200 μ g of HEL, whereas a similar dose of HEL alone does not (Table II), and there is no difference in the amount of circulating HEL in either case (Table III), then CTX must be inducing Ab production by means other than increased HEL uptake.

The s.c. immunization of mice with Ag was not augmented by CTX, whereas the oral immunization was, as indicated by Ab response and histamine release. Mice immunized orally with HEL and CTX were routinely sensitized, even though their circulating HEL concentrations were small relative to s.c. mice. This implies that the intestinal immune response is much more susceptible to CTX adjuvant effects. One could make two independent

FIGURE 8. Oral immunization with HEL and CTX or s.c. with HEL sensitizes intestinal tissue for HEL-specific anaphylactic response. **A.** Mean Δ Isc ($\pm$ SEM) measures of intestinal secretory response, of tissues tested with HEL challenge on the serosal side (hatched bars; individual, $\blacktriangle$) or the epithelial side (open bars; individual, $\bullet$). Tissues were from groups of five to eight mice immunized orally twice with 10 μ g CTX and 300 μ g HEL (CTX-HEL), orally with 300 μ g HEL alone (HEL), or orally with 10 μ g CTX alone (CTX), or immunized s.c. with 30 μ g HEL (HEL s.c.), or not immunized (N). **B.** Representative tracings of chart recorder lines for Isc response by intestinal tissues from one mouse of each group described in A. They are: a) CTX-HEL, b) HEL s.c., c) CTX, or d) HEL. Time scale is left to right as shown. Isc response occurs after introduction of HEL (arrow).



but not mutually exclusive arguments concerning the special adjuvant activities of CTX given orally. First, the adjuvant effect could be attributed to some combination of effects by CTX on B cells (16, 43) and T helper cells (15) or on APCs such as macrophage (10, 11), or perhaps epithelial cells (14). In this regard, our data do not help us determine whether all of the response is generated by mucosal lymphocytes in the Peyer's patch, mesenteric lymph nodes, or lamina propria. Experiments designed to locate B cells that produce anti-HEL IgE Ab in Peyer's patch or mesenteric lymph nodes should help answer this question. The alternative argument is that protein Ag may be manipulated differently or sequestered in the intestinal mucosa and that circulating quantities of HEL do not reflect the true effective dose of Ag to the intestinal mucosa in the presence of CTX. For instance, there is some evidence that cultured monolayers of epithelial cells have increased permeability for protein Ags in the presence of CTX (44). Perhaps CTX allows increase Ag uptake to the lamina propria and Ag is efficiently used or sequestered in this site.

Our results imply that the use of CTX to augment a response to protein Ag in vaccines for humans may result in IgE production and a hypersensitive state, that could prove life-threatening. It is likely that at least some individuals are genetically predisposed to a large Ab response or to a T helper cell response that is dominated by Th2 cytokines, for any given protein Ag. These individuals may develop a hypersensitive state when exposed to oral vaccines that include CTX used as a mucosal adjuvant. In this regard, it is important to note that some vaccine work has focused on the use of the CTB subunit of the holotoxin as an adjuvant for IgA Ab production against unrelated

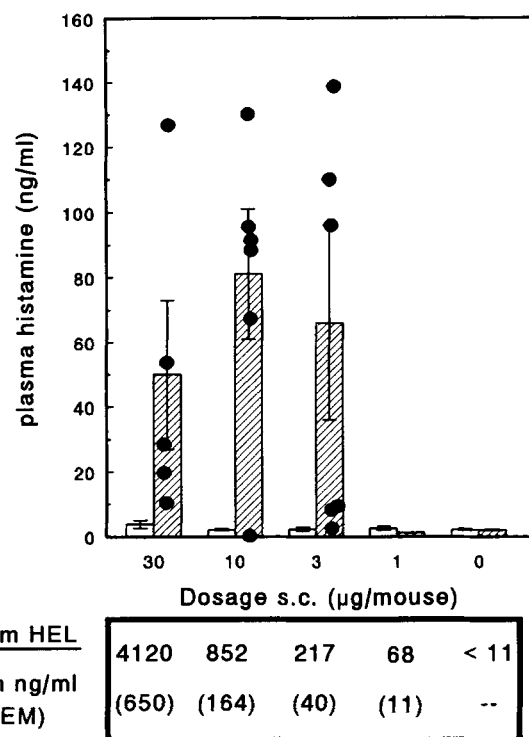


FIGURE 9. The upper figure shows plasma histamine levels in mice immunized s.c. with HEL alone at the indicated dosages (x-axis). Mean values are shown for prebleeds (open bars $\pm$ SEM) and bleeds at 10 min post challenge (hatched bars $\pm$ SEM). Individual values ($\bullet$) are shown for groups of five to six mice, in groups with a detectable response. The lower figure indicates the mean serum HEL concentration with SEM in parenthesis, at 15 min after the first s.c. injection for each group of mice.

Table III. No effect of CTX on HEL uptake to circulation

Treatment	Positive Serum HEL ^a Positive/Total	Mean Serum HEL ^b ng/ml (SEM)	Range ng/ml	Number >200 ng/ml
Expt. 1				
Oral HEL (1 mg)	6/10	126 (54) ^c	10–335	2/6
Oral HEL (1 mg) plus CTX	7/10	120 (51)	13–296	3/7
Expt. 2				
Oral HEL (200 µg)	6/10	55 (33)	11–172	0/6
Oral HEL (200 µg) plus CTX	5/10	45 (28)	11–144	0/5

^a Limit of detection was 8 ng/ml (at 1/10 dilution) in expt. 1 and 2 ng/ml in expt. 2. All prebled sera were negative. Sera were taken at 3 h for expt. 1 and at 2 or 4 h (pooled data) for expt. 2.

^b Mean calculated for positive sera only.

^c One serum in HEL alone group was excluded as anomalously high (2 µg/ml).

protein Ags, with some success, but only when the protein Ag was directly conjugated to CTB (6, 45, 46). However, conflicting reports indicate that CTB has no adjuvant effects when given orally, unless given with a least small quantities of whole holotoxin, especially when the recipient animal does not make a strong response to CTX itself (7, 47). Our results indicate that CTB does not potentiate allergic sensitivity to mice and that the presence of the A chain is necessary, both for a strong Ab response (IgA included) and to sensitize the mice. CTB may work better as an adjuvant when administered intranasally (7, 48). In addition, in vitro studies indicate that CTB has the same IgG1- and IgE-potentiating function as CTX, but to a lesser degree (16, 49). Additional experiments comparing the effects of CTX and CTB on induction of IgE Ab to protein Ags during oral immunization seem warranted. It will be especially important to separate the adjuvant effects on IgA production from those that promote IgE production.

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