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Cholera Toxin as a Mucosal Adjuvant: Effects of *H-2* Major Histocompatibility Complex and *lps* Genes

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In previous studies we found that cholera toxin (CT) can act as a mucosal adjuvant; i.e., it can stimulate an intestinal secretory immunoglobulin A (S-IgA) response to an unrelated protein antigen when both are fed together to mice. The purpose of this study was to determine whether the mucosal adjuvanticity of CT is restricted by either *H-2* major histocompatibility complex or *lps* genes by using congenic inbred strains that differ at only a single genetic locus. Groups of five mice each were fed saline, CT (10 μg), keyhole limpet hemocyanin (KLH) (5 mg), or both CT and KLH on four different days, and samples of intestinal secretions and plasma were obtained 1 week after the last feeding. In the mice fed both CT and KLH, the intestinal S-IgA anti-KLH response was higher in $H\text{-}2^b$ congenic strains than in $H\text{-}2^k$ congenic strains, and in addition there was a highly significant positive correlation between the intestinal S-IgA anti-KLH and S-IgA anti-CT responses in the intestinal secretions of individual mice. Similarly, in the *lps* congenic strains, mice of the endotoxin-responsive strain that were fed both CT and KLH had substantially higher S-IgA and plasma IgG responses to KLH than did mice of the endotoxin-unresponsive strain. The effect of CT on the induction of oral tolerance to KLH in the *H-2* congenic strains was also examined. In contrast to the results above, the abrogation of oral tolerance to KLH by CT occurred in all strains regardless of *H-2* haplotype. Similarly, the adjuvant effect of CT on plasma IgG anti-KLH responses after both were given together intraperitoneally was not restricted by *H-2*. I conclude that the mucosal adjuvanticity of CT is influenced by both the *H-2* and *lps* genetic loci and that it appears to depend on a vigorous mucosal immune response to CT itself.

Cholera toxin (CT) is a protein enterotoxin composed of two subunits, the adenylate cyclase activating, or A, subunit and the binding, or B, subunit (2). The latter is a pentameric homodimer that binds to G_{M1} ganglioside, a membrane glycolipid present on all nucleated cells (6). CT is a very potent mucosal immunogen, and although the mechanism of its potency remains undefined, both the A and B subunits seem to play a role (25). One factor involved in the degree of its immunogenicity in mice is the genetic background of the mouse strain. In previous studies, we found different inbred strains of mice vary widely in their immunologic response to CT and that this genetic component could be mapped to the I-A subregion of the major histocompatibility complex (MHC) (10, 14).

A second unusual property of CT is that of mucosal adjuvanticity; i.e., when a second, unrelated antigen is coadministered with CT into the intestine, a secretory immunoglobulin A (S-IgA) response to the unrelated antigen is generated, even though no such response occurs when the antigen is fed by itself (8, 21). At the same time, CT abrogates the oral tolerance that otherwise occurs after the feeding of the other, unrelated protein antigen (8). Again, the mechanism of this adjuvant effect is unclear; however, certain parameters have been defined; e.g., the adjuvant effect is seen only if CT is given with the other antigen by the same route and at the same time (21).

The present experiments were prompted by the results of some experiments in which there was little or no mucosal adjuvant effect of CT in an inbred strain that was an immunologic low responder to CT itself (unpublished data). These results suggested that there are genetic influences on the mucosal adjuvanticity of CT. In the data reported here, the mucosal adjuvanticity of CT was studied in congenic inbred strains of mice that differ at only a single genetic locus, i.e., *H-2* or *lps*. The latter locus is of interest because lipopolysaccharide is abundant in the gut, has potent immunologic effects including adjuvant activity (18), and is likely to be present continuously in gut-associated lymphoid tissue, i.e., simultaneously with CT or other antigens given into the intestine.

MATERIALS AND METHODS

Animals. C3H/HeN mice were obtained from Frederick Cancer Research Center, Frederick, Md. All other strains were obtained from the Jackson Laboratory, Bar Harbor, Maine. Most of the mice used were 6- to 12-week-old females.

Schedule of immunization and sampling. CT was obtained from Sigma Chemical Co., St. Louis, Mo., and keyhole limpet hemocyanin (KLH) was obtained from Calbiochem-Behring, San Diego, Calif. Groups of five mice of each strain were given 5 mg of KLH with or without 10 μg of CT in 0.5 ml of 0.2 M $NaHCO_3$ intragastrically (i.g.) by using feeding needles (G. Tiemanns and Sons, Long Island City, N.Y.) with priming doses on days 0 and 1 and booster doses on days 14 and 15. Samples of plasma and intestinal secretions were taken before feeding and on days 21 and 22, respectively. The mice were then parenterally immunized with 1 μg of KLH in 20% alum intraperitoneally (i.p.) on days 22 and 50. Samples of plasma were taken on day 57. Each mouse was ear tagged, and samples from each were individually identified.

Collection of samples. Blood was obtained from the retro-orbital plexus of ether-anesthetized mice by using heparinized capillary tubes. After transfer to small plastic centrifuge tubes and centrifugation for 5 min at 11,000 × *g* in a microcentrifuge, the plasma was aspirated off and stored at −20°C until assay. Intestinal secretions were collected by using a lavage technique which has been described (11).

Briefly, the mice were placed on a square (12 by 12 cm) of galvanized wire mesh which was in turn placed on a plastic petri dish (100 by 15 mm) containing 3 ml of a solution of protease inhibitors. The mice were kept on the wire mesh by inverting a 600-ml glass beaker over the mice. Four doses of 0.5 ml of the lavage solution were given i.g. at 15-min intervals by using a blunt-tipped feeding needle. The lavage solution consisted of 25 mM NaCl, 40 mM Na_2SO_4, 10 mM KCl, 20 mM $NaHCO_3$, and 48.5 mM polyethylene glycol (average molecular weight = 3,350). Thirty minutes after the last doses of the lavage solution, the mice were given 0.1 mg of pilocarpine i.p. A discharge of intestinal contents occurred regularly over the next 10 to 20 min. The protease inhibitor solution was a mixture of soybean trypsin inhibitor, EDTA, and phenylmethylsulfonyl fluoride as described previously (11). The samples were stored frozen at −20°C until assay.

Measurement of antibody. Plasma IgG and IgA and intestinal S-IgA anti-KLH and anti-CT were measured by enzyme-linked immunosorbent assay (ELISA) as described elsewhere (8). Briefly, antigen was coated on the wells of a 96-well polystyrene Immulon plate (Dynatech, Rockville, Md.). After an incubation with sample or standard mouse antibody, the wells were then incubated with rabbit anti-mouse IgG or rabbit anti-mouse IgA, followed by an incubation with sheep anti-rabbit globulin coupled to alkaline phosphatase. Finally, 0.1 ml of *p*-nitrophenyl-phosphate (1 mg/ml; Sigma) in 10% diethanolamine buffer at pH 9.8 was added to each well, and the color development after a 30-min incubation at room temperature was measured at 405 nm in a MR580 micro-ELISA autoreader (Dynatech Instruments, Santa Monica, Calif.). A standard curve was constructed for each assay, and the values of the samples were interpolated by using a program based on Rodbard's four-parameter logistic model (29) on an Apple II Plus microcomputer (Cupertino, Calif.). As a standard for anti-CT antibody, a reference hyperimmune serum was used, and the antibody concentration was expressed in units of standard activity: 1 U of anti-CT antibody is defined as that amount which gave an A_{405} equivalent to a 10^6 dilution of the reference anti-CT antiserum. As a standard for anti-KLH, an affinity-purified antibody was used and the results were expressed as micrograms per milliliter.

Total IgA in intestinal secretions was measured by sandwich ELISA (11). The wells of the Immulon plate were coated with affinity-purified goat anti-mouse IgA (Cappel Laboratories, Pittsburgh, Pa.). After the incubation of samples and standards, the wells were sequentially incubated with a rabbit anti-mouse IgA and sheep anti-rabbit globulin coupled to alkaline phosphatase. A quantitated reference mouse serum (Meloy Laboratories, Springfield, Va.) was used as a standard. All the other aspects of the assay are the same as those described above for the measurement of specific antibody.

Statistics. Antibody levels were converted to logarithmic values for calculation of geometric means and standard errors. S-IgA levels were expressed as units per milliliter of secretions recovered or as units per microgram of total IgA. The latter value was obtained by dividing the geometric mean IgA anti-CT per milliliter by the micrograms per milliliter of total IgA in the same sample. The significance of correlation between the levels of S-IgA anti-CT and S-IgA anti-KLH in the intestinal secretions of individual mice was tested by linear regression on a Macintosh microcomputer using a statistical program (StatWorks; Heyden and Son, Inc., Philadelphia, Pa.).

TABLE 1. Intestinal S-IgA responses to KLH and CT after oral immunization of *H-2* congenic strains

Strain	Feeding[a]	S-IgA anti-KLH (ng/ml)[b]	S-IgA anti-CT (U/ml)[b]
B10 (*H-2*^{b})	—	<2	<100
	CT	<2	12,770 (2.00)
	KLH	<2	213 (2.08)
	KLH + CT	37.2 (1.54)	8,808 (1.54)
B10.BR (*H-2*^{k})	—	2.2 (1.25)	<100
	CT	2.1 (1.55)	<100
	KLH	<2	<100
	KLH + CT	2.2 (2.19)	172 (2.52)
C3H.SW (*H-2*^{b})	—	12.2 (2.48)	155 (1.82)
	CT	4.2 (3.20)	105,156 (4.28)
	KLH	5.5 (2.18)	<100
	KLH + CT	68.1 (2.36)	126,994 (1.32)
C3H/HeN (*H-2*^{k})	—	6.3 (2.36)	<100
	CT	3.8 (1.28)	1,677 (1.98)
	KLH	5.6 (1.30)	<100
	KLH + CT	24.1 (2.55)	3,818 (1.86)

[a] Groups of five mice were given $NaHCO_3$ alone (—), 5 mg of KLH, 10 μg of CT, or both KLH plus CT at the same doses i.g. on days 0, 1, 14, and 15. Intestinal lavage samples were obtained on day 21 and analyzed for antibody.

[b] Data shown are geometric means (geometric standard deviations in parentheses) on day 21.

RESULTS

Effect of *H-2* MHC locus on mucosal adjuvanticity of CT. Two sets of H-2 congenic mice were used in these experiments, the first set being B10 (*H-2*^{b}) and B10.BR (*H-2*^{k}) and the second set being C3H.SW (*H-2*^{b}) and C3H/HeN (*H-2*^{k}). Groups of five mice each were given saline, 10 μg of CT, 5 mg of KLH, or both KLH and CT i.g. The antibody levels that were present in intestinal lavage and plasma are shown in Tables 1 and 2, respectively. The intestinal S-IgA responses to CT varied among the congenic inbred strains as had been previously reported, being high in *H-2*^{b} strains and low in *H-2*^{k} strains regardless of the other genes of the background strain (Table 1). Again, as previously reported, the feeding of KLH alone did not result in an intestinal or plasma antibody response to this protein. Importantly, in the groups fed both KLH and CT, the intestinal S-IgA anti-KLH response was enhanced by CT only in the *H-2*^{b} strains (B10 and C3H.SW), whereas in the *H-2*^{k} strains (B10.BR and C3H/HeN) there was little or no adjuvant effect of CT evident (Table 1). The plasma IgG responses of the groups fed KLH plus CT showed a similar effect, with plasma IgG anti-KLH being markedly enhanced in the B10 strain but not in B10.BR (Table 2). Both C3H strains had weak plasma IgG responses to KLH, but the increase over background in C3H.SW (*H-2*^{b}) was slightly greater than that in C3H/HeN (*H-2*^{k}).

Intestinal samples from individual mice of the different *H-2* congenic strains were separately identified. Figure 1 shows the S-IgA anti-KLH plotted against S-IgA anti-CT present in the day 21 intestinal samples of each mouse of the groups fed KLH and CT. There is a highly significant correlation as determined by linear regression analysis (R = 0.69, $P < 0.01$).

Effect of *lps* gene locus on mucosal adjuvanticity of CT. In these experiments, *lps* congenic mice on the C57BL/10 (*H-2*^{b}) background were used, with C57BL/10 SnJ being endotoxin responsive (*lps*$^+$) and C57BL/10 ScN being endo-

TABLE 2. Plasma IgG responses to KLH and CT after oral immunization of *H-2* congenic strains

Strain	Feeding[a]	Plasma IgG anti-KLH (ng/ml)[b]	Plasma IgG anti-CT (U/ml)[b]
B10 (*H-2*^{b})	—	<0.1	28 (1.43)
	CT	<0.1	18,700 (1.21)
	KLH	0.2 (2.96)	26 (1.18)
	KLH + CT	59.9 (2.37)	8,400 (1.48)
B10.BR (*H-2*^{k})	—	0.1 (1.21)	<3
	CT	0.2 (1.14)	7 (2.95)
	KLH	0.3 (3.76)	<3
	KLH + CT	6.2 (10.47)	13 (5.62)
C3H.SW (*H-2*^{b})	—	0.1 (1.45)	<3
	CT	0.2 (1.62)	10,900 (1.24)
	KLH	0.3 (2.38)	<3
	KLH + CT	2.0 (6.05)	6,790 (1.25)
C3H/HeN (*H-2*^{k})	—	0.2 (1.62)	3 (1.35)
	CT	0.3 (1.79)	73 (1.71)
	KLH	0.3 (3.04)	6 (1.79)
	KLH + CT	1.5 (6.35)	12 (2.46)

[a] Groups of five mice were given $NaHCO_3$ alone (—), 5 mg of KLH, 10 μg of CT, or both KLH plus CT at the same doses i.g. on days 0, 1, 14, and 15, and plasma was obtained on day 22 for antibody measurements.

[b] Data shown are geometric means (geometric standard deviations in parentheses) on day 22.

toxin nonresponsive (*lps*$^-$). Groups of mice were fed saline, CT, KLH, or both i.g. in a fashion identical to that for the *H-2* congenic inbred strains. The intestinal and plasma anti-CT responses were strong and comparable in both of these strains (Table 3). Neither strain developed a significant anti-KLH response when fed KLH alone. Interestingly, in the group fed both KLH and CT, mice of the *lps*$^+$ strain, C57BL/10 SnJ, had a substantially higher S-IgA and plasma IgG responses to KLH than did mice of the *lps*$^-$ congenic strain, C57BL/10 ScN.

Effect of *H-2* MHC locus on abrogation of oral tolerance to KLH by CT. After the oral feeding, the *H-2* congenic strains were immunized i.p. with 1 μg of KLH in alum. Again, the MHC genetic restriction of the plasma IgG anti-CT response was clearly demonstrated in the group fed either CT alone or both KLH and CT, with *H-2*^{b} mice having high anti-CT responses and *H-2*^{k} mice having low anti-CT responses (Table 4). Groups fed KLH and then immunized i.p. with KLH had a markedly reduced plasma IgG anti-KLH response compared with that of control mice fed saline and then immunized i.p. with KLH, consistent with the induction of oral tolerance by the feeding of KLH. In marked contrast, groups fed KLH plus CT and then immunized i.p. with KLH had an augmented plasma IgG anti-KLH response. Such abrogation of oral tolerance to KLH by coadministration of CT was unaffected by the *H-2* haplotype of the mice.

Effect of *H-2* MHC locus on parenteral adjuvanticity of CT. Groups of mice from the *H-2* congenic strains were injected i.p. with either 100 μg of soluble KLH alone or 100 μg of KLH–0.2 μg of CT on days 0 and 15. Plasma was obtained on days 14 and 21 for measurement of primary and secondary plasma IgG anti-KLH antibody responses. An adjuvant effect of the CT on plasma IgG anti-KLH responses was found in both sets of *H-2* congenic inbred strains (Table 5). Moreover, the degree of the adjuvant effect of CT given parenterally was comparable among the strains and did not vary according to *H-2* haplotype.

DISCUSSION

Despite the large amounts of IgA produced daily at mucosal surfaces (3), it is remarkably difficult to generate at will an intestinal IgA response to protein antigens. Not only does the feeding of protein antigens fail generally to stimulate an intestinal S-IgA response, it frequently results in a state of oral tolerance; i.e., there is a markedly lower response to subsequent parenteral immunization with that same antigen (5, 31). This is a particular problem when one is attempting to develop a secretory immune response against protein antigens of microbial pathogens. The pattern of response to the feeding of KLH, a good parenteral immunogen, is typical of the pattern of response to the feeding of many, if not most, protein antigens, i.e., the induction of tolerance but not an intestinal S-IgA response (Tables 1 and 4) (8, 9). A striking exception to this pattern of response is manifested by CT, which both stimulates a strong mucosal S-IgA response and does not generate oral tolerance when fed to mice (8, 9). The immune responses of the groups of mice fed KLH alone and fed CT alone in the present study are very similar to the results that were obtained in previous reports. They were included in this study as controls against which to compare the results obtained when both KLH and CT were fed together to mice of congenic strains that differ at only a single genetic locus.

In previous studies, we reported that coadministration of KLH and CT into the intestines of mice dramatically altered the pattern of response to KLH by abrogating oral tolerance and stimulating an intestinal S-IgA anti-KLH response (8). This observation has been repeated now for multiple antigen systems (1, 19, 21), and the use of CT as a mucosal adjuvant is emerging as an important strategy for mucosal immunization and oral vaccine development (7). There are a number of variables that have been defined and need to be considered in such usage of CT as discussed below. Whether genetic factors influence CT's adjuvanticity has been previously examined in two previous studies. One study found variable but quantitative differences in several *H-2* disparate inbred strains of mice, but the interpretation was clouded by

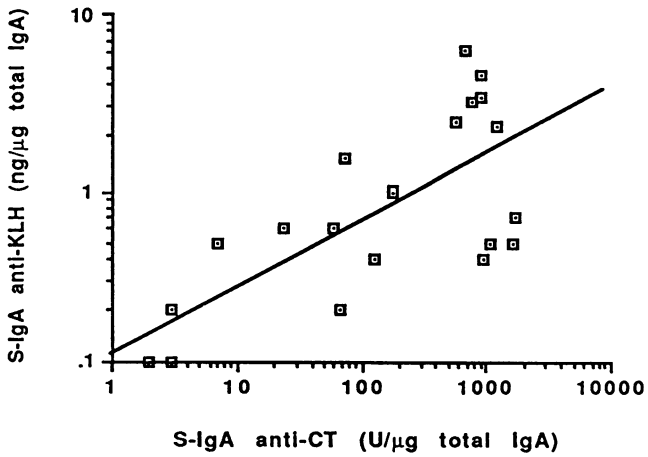

FIG. 1. S-IgA anti-KLH versus S-IgA anti-CT in intestinal secretions of individual mice of *H-2* congenic strains that were fed both KLH and CT. There is a significant positive correlation (R = 0.69, $P < 0.01$) between the levels of the two antibodies.

TABLE 3. Intestinal and plasma antibody response after antigen feeding of *lps*$^+$ and *lps*$^-$ congenic strains

Strain	Feeding[a]	Intestinal S-IgA response[b]		Plasma response[b]			
		Anti-KLH (ng/ml)	Anti-CT (U/ml)	Anti-KLH (μg/ml)		Anti-CT (U/ml)	
				IgG	IgA	IgG	IgA
C57BL/10 SnJ (*lps*$^+$)	—	<3	<200	0.52 (1.32)	<0.2	<2.5	<2.5
	CT	<3	17,084 (2.23)	0.49 (1.54)	0.25 (1.41)	13,802 (1.51)	281 (1.42)
	KLH	<3	<200	0.47 (1.22)	<0.2	3.2 (2.64)	3.3 (1.80)
	KLH + CT	78 (1.98)	13,940 (1.81)	11.0 (1.45)	0.38 (1.54)	11,008 (1.72)	206 (1.26)
C57BL/10 ScN (*lps*$^-$)	—	<3	<200	0.44 (1.14)	<0.2	3.9 (2.28)	2.6 (1.45)
	CT	<3	24,613 (1.66)	0.38 (1.49)	<0.2	9,230 (1.45)	374 (1.48)
	KLH	4.9 (1.99)	<200	0.42 (1.43)	0.24 (1.25)	<2.5	<2.5
	KLH + CT	10.8 (1.68)	11,163 (3.12)	0.83 (1.69)	0.48 (1.40)	5,935 (1.29)	197 (1.74)

[a] Groups of five mice were given $NaHCO_3$ alone (—), 5 mg of KLH, 10 μg of CT or both i.g., as shown, on days 0, 1, 14, and 15, and intestinal secretions and plasma were obtained on days 20 and 21, respectively, for measurement of antibody.
[b] Data are shown as geometric means (geometric standard deviations in parentheses) on day 21 (S-IgA) and day 22 (plasma).

differences in multiple background genes among the strains (32). The second found no evidence of *H-2* restriction of adjuvanticity of CT-B when it was coadministered in high concentrations, along with influenza virus hemagglutinin, into the nose (16). The present study used congenic inbred strains of mice, focusing on two well-defined genetic loci, *H-2* and *lps*. The key finding is that the mucosal adjuvanticity of CT was notably influenced by each of these genetic loci: mice that were of the *H-2*^{b} haplotype and that were *lps* responsive exhibited a much more pronounced mucosal adjuvant effect of CT. In addition, in mice of different *H-2* haplotypes, the intestinal S-IgA anti-CT and S-IgA anti-KLH responses of individual mice showed a strong positive correlation, indicating that the adjuvanticity of CT is related to its immunogenicity. This finding may have significant implications for the use of CT as a mucosal adjuvant in outbred populations. One would expect that CT will not be effective as a mucosal adjuvant in outbred individuals that are poor responders to CT itself. The exact proportion of individuals that would fit this category at present is unknown. In humans and most other outbred populations, this proportion should not be very large, because CT is known to be an effective mucosal immunogen in most species (17, 26). This relationship between the immunogenicity and adjuvanticity of CT may limit its effectiveness as a mucosal adjuvant in previously immunized individuals, but this theory needs to be directly examined.

The genetic background of the individual can now be added to the important parameters that define the mucosal adjuvanticity of CT. Other parameters that have been shown to be important include dose, route, and timing of the CT delivery (21). Most studies have utilized 1 to 20 μg of CT per dose in mice with lesser amounts being relatively ineffective. Both CT and antigen must be given by the same route, i.e., into the intestine, and optimally at the same time. Delivery of CT hours apart from the antigen is ineffective (21). This relatively small window of adjuvanticity may explain why CT administration does not cause a general increase in S-IgA levels or the development of S-IgA antibodies to common

TABLE 4. Abrogation of oral tolerance to KLH by coadministration of CT in *H-2* congenic strains

Strain	Feeding[a]	Plasma IgG anti-KLH[b] (μg/ml)	Plasma IgG anti-CT[b] (10^3 U/ml)
B10 (*H-2*^{b})	—	366 (2.30)	50 (1.50)
	CT	329 (2.59)	8,660 (1.61)
	KLH	8 (1.31)	51 (1.24)
	KLH + CT	1,350 (2.37)	3,310 (1.68)
B10.BR (*H-2*^{k})	—	165 (1.92)	<3
	CT	89 (1.14)	56 (1.08)
	KLH	5 (5.43)	<3
	KLH + CT	1,105 (2.25)	11 (3.02)
C3H.SW (*H-2*^{b})	—	1,628 (1.90)	<3
	CT	1,711 (5.05)	6,940 (1.29)
	KLH	388 (2.69)	<3
	KLH + CT	2,648 (1.38)	3,990 (1.74)
C3H/HeN (*H-2*^{k})	—	1,432 (1.45)	15 (1.41)
	CT	1,543 (1.16)	91 (2.65)
	KLH	9 (6.93)	9 (3.04)
	KLH + CT	2,409 (1.62)	43 (1.79)

[a] Groups of five mice were fed with $NaHCO_3$ alone (—), KLH, CT, or KLH plus CT at the same doses described in Table 1; then, they were immunized parenterally with 1 μg of KLH in alum i.p. on days 22 and 50. Plasma was obtained on day 57 for antibody measurement.
[b] Data shown are geometric means (geometric standard deviations in parentheses) on day 57.

TABLE 5. Effect of parenteral injection of KLH versus KLH plus CT on the plasma anti-KLH response in *H-2* congenic strains

Strain	Injection[a]	Plasma IgG anti-KLH (μg/ml)[b]	
		Primary (day 14)	Secondary (day 21)
B10 (*H-2*^{b})	KLH	21 (1.59)	233 (1.28)
	KLH + CT	39 (1.28)	1,117 (1.23)
B10.BR (*H-2*^{k})	KLH	25 (1.88)	534 (1.41)
	KLH + CT	32 (1.79)	2,511 (1.11)
C3H.SW (*H-2*^{b})	KLH	1 (1.72)	25 (1.95)
	KLH + CT	65 (1.50)	2,796 (1.35)
C3H/HeN (*H-2*^{k})	KLH	3 (2.09)	12 (1.81)
	KLH + CT	34 (1.33)	538 (1.36)

[a] Groups of five mice were injected i.p. with 100 μg of soluble KLH or 100 μg of KLH plus 0.2 μg of CT on days 0 and 15, and plasma was obtained on days 14 and 21 for measurement of antibody.
[b] Data shown are geometric means (geometric standard deviations in parentheses). Preimmunization plasma antibody level in all mice was 0.1 μg/ml or less.

dietary protein antigens (24). These parameters do indicate that adjuvanticity is due to a transient action on the local mucosa, presumably by effects in the lymphoid follicles of gut-associated lymphoid tissue; however, the exact site of its adjuvant effect has yet to be experimentally demonstrated.

The genetic effects on CT's mucosal adjuvanticity for S-IgA responses in the present study contrast with the lack of either genetic locus to affect the ability of CT to abrogate oral tolerance or to act as a parenteral immunogen. However, both of the latter results may reflect quantitative rather than qualitative differences from its mucosal effects. Oral tolerance has been shown to involve the generation of $CD8^+$ suppressor T cells (28). It is quite possible that the inhibition of this T-cell subset might be very sensitive to the effects of CT. Indeed, in in vitro studies we have found that both CT and CT-B subunit are potent inhibitors of T-cell activation and that the $CD8^+$ T-cell subset is more susceptible to this inhibition than is the $CD4^+$ T-cell subset (13). These in vitro studies also fail to show any effects of the *H-2* locus on such inhibition, but whether these observations relate to the present findings is unclear. The lack of genetic effect on the parenteral adjuvanticity of CT might be interpreted as indicating that the mucosal and parenteral adjuvant effects involve different mechanisms. However, some genetic restrictions can be overcome by high doses of antigen and the groups receiving parenteral KLH plus CT very likely received a great deal more of both KLH and CT than are delivered to mucosal lymphoid tissues after i.g. feeding, given the anticipated degradation of these proteins in the gut by acid, proteases, and bile; thus, a genetic restriction might have been evident at more limited doses of adjuvant and antigen. This point may relate also to the discordance of the present results with those of Hirabayashi et al., who gave high concentrations of CT-B as an adjuvant into the nose (16). The effective dose by this route was also likely much higher than when the same amounts are given i.g. The lack of an *H-2* restriction when the nasal route was used may thus correspond to the lack of *H-2* restriction after parenteral administration in the present studies. If so, this implies that the *H-2* restriction with the mucosal route might be overcome by greater amounts of CT.

The mechanism of CT's mucosal adjuvanticity is likely to be complex and multifactorial. A number of possibilities have been proposed, including an increase in antigen-presenting cell function in the mucosa (4, 20) and an enhanced switching of IgC_H genes to favor both IgG and IgA (23). Interestingly, the systems that have demonstrated these effects have utilized lipopolysaccharide as a cofactor to stimulate cells in vitro. The observation here that the *lps* locus is an important determinant of CT's adjuvanticity supports these potential mechanisms. A third mechanism that has been proposed is that of an increased permeability of the intestinal mucosa, presumably allowing antigen to penetrate (22). This mechanism might also show evidence of *H-2* restriction in that G_{M1} ganglioside expression (15, 30) and the intestinal fluid accumulation after CT administration (27) have been found to be genetically restricted by a locus close to *H-2*. Moreover, intestinal fluid accumulation after giving CT is higher in mice of the $H\text{-}2^b$ haplotype than in mice of the $H\text{-}2^k$ haplotype, similar to the results found here for CT's adjuvanticity. A fourth mechanism that has been proposed is the inhibition of suppressive mechanisms in the mucosa-associated lymphoid tissue (12), has been discussed above. The influences of the *H-2* and *lps* genes on the mucosal adjuvanticity of CT should help to sort out which potential mechanisms are more biologically relevant, because those relevant mechanisms should exhibit these same genetic restrictions.

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