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Oral Administration of a Streptococcal Antigen Coupled to Cholera Toxin B Subunit Evokes Strong Antibody Responses in Salivary Glands and Extramucosal Tissues

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Generation of local and systemic immune responses by the oral administration of antigens is frequently inefficient, requiring large quantities of immunogens and yielding only modest antibody responses. In this study, we have demonstrated that oral administration of microgram amounts of *Streptococcus mutans* protein antigen I/II covalently coupled to the B subunit of cholera toxin elicits vigorous mucosal as well as extramucosal immunoglobulin A and G antistreptococcal antibody responses in mice. These responses were manifested by the presence of large numbers of antibody-secreting cells in salivary glands, mesenteric lymph nodes, and spleens and by the development of high levels of circulating antibodies. This novel immunization strategy may find broad application in the construction of oral vaccines for the control of infectious diseases caused by pathogens encountered at mucosal and extramucosal sites.

Generation of a protective antibody response at mucosal surfaces, where many significant infections begin, is not readily achieved by the conventional route of parenteral injection, although this is usually effective in eliciting circulating antibodies (11, 23, 25). In contrast, oral administration of antigens may result in the concomitant expression of antibody responses in various mucosal tissues and secretions, usually without a pronounced systemic antibody response (8, 20, 21, 23, 25). This apparent compartmentalization of systemic and secretory humoral immune responses may explain why parenteral vaccines are only partly effective against mucosal pathogens.

It is believed that ingested antigens are absorbed and processed by specialized cells, including epithelial enterocytes and Peyer's patch M cells, in the gut-associated lymphoid tissue (26). After interaction with accessory cells and cognate helper T cells, antigen-sensitized B lymphocytes disperse (while differentiating) via mesenteric lymphatics, the thoracic duct, and the circulation to various mucosal tissues, where they mature into plasma cells secreting antibodies primarily of the immunoglobulin A (IgA) isotype (2, 13). This hypothesis has contributed to the concept of a common mucosal immune system (16, 36) and has provided a rational basis for the development of oral vaccines against mucosal pathogens (20).

However, stimulation of gut-associated lymphoid tissue by the oral consumption of most nonviable antigens is often inefficient, requiring large (milligram) quantities of immunogens and yielding, if any, modest antibody responses (4, 11, 20–25). A notable exception is cholera toxin (CT), which is a potent enteric immunogen (27). This is due, to a large extent, to the ability of CT to bind avidly to G_{M1} ganglioside on cell surfaces, a property ascribed to its B subunit (9). More recently, CT has been shown to exert potent adjuvant effects in mice on gut immune responses to unrelated antigens presented orally (7, 15, 24).

Since oral delivery of vaccines is a convenient route of immunization, we have attempted to stimulate the common mucosal immune system with small doses of a streptococcal antigen by using the B subunit of CT (CTB) as carrier protein (19) and subclinical doses of free CT as adjuvant. This streptococcal antigen, designated antigen I/II (Ag I/II), is a prominent surface protein of the cariogenic organism *Streptococcus mutans* (28). On parenteral immunization, Ag I/II readily induces serum antibody responses in rodents and primates (14, 29) and also confers partial immunity against dental caries in monkeys (14). In the latter study, protection was associated with the presence of IgG antibodies in serum and gingival fluid (a transudate of presumptive serum origin) but not with salivary antibodies (14). In contrast, multiple administrations of large doses of *S. mutans* antigens given orally in rodents and in humans induce secretory IgA antibody responses in saliva without pronounced antibody responses in serum (4, 21, 22, 34). Most importantly, such oral immunization schemes have been shown to confer partial immunity to dental caries in rats (22) and also to interfere with colonization of tooth surfaces by *S. mutans* in humans (21, 34). Because of the close anatomical relations between enamel tooth surface with saliva containing predominantly secretory IgA antibodies and gingival fluid containing serum-derived IgG antibodies, two mechanisms of antibody-mediated protection may both be operative (17). In fact, early immunization regimens involving local injection of *S. mutans* antigens in Freund adjuvant into the salivary glands of rodents have been shown to induce both salivary and serum antibody responses and to confer a high degree of protection against *S. mutans*-induced dental caries (18, 33). Such immunization protocols, although providing promising results for the development of a caries vaccine, cannot be considered for human use.

A major goal in our studies is to develop an oral immunization scheme which induces both types of antibody responses, without requiring massive doses of immunogen. Here we show that intragastric administration to mice of microgram amounts of a conjugate consisting of Ag I/II covalently coupled to CTB elicits strong IgA and IgG

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antibody responses in mucosal tissues, including salivary glands, as well as vigorous serum antibody responses.

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MATERIALS AND METHODS

Immunogens. CT was obtained from List Biological Laboratories, Inc. (Campbell, Calif.). CTB, free of toxic activity, was prepared at the Institut Merieux, Lyon, France, according to previously published purification protocols (10, 35).

S. mutans Ag I/II was purified as described in detail elsewhere (28). CTB and Ag I/II were each coupled to *N*-succinimidyl-(3-[2-pyridyl]-dithio)propionate (SPDP) (1) at molar ratios of 1:30 and 1:10, respectively, according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). The preparations were then freed of unreacted SPDP by gel filtration through Sephadex G25 columns (Pharmacia). The Ag I/II derivative was reduced for 20 min at ambient temperature with 50 mM dithiothreitol in 0.1 M sodium acetate buffer–0.1 M NaCl (pH 4.5) and gel filtered through a Sephadex G25 column equilibrated with 0.1 M sodium phosphate buffer–0.1 M NaCl (pH 7.5). The resulting Ag I/II preparation was then incubated in equimolar proportions with the CTB derivative for 16 h at room temperature. The mixture was finally dialyzed extensively against 0.01 M phosphate buffer–0.15 M NaCl (pH 7.4). The resulting conjugate was shown to contain G_{M1} ganglioside-binding capacity and to retain both CTB and Ag I/II serological reactivities by enzyme-linked immunosorbent assay (ELISA) using G_{M1} (Sigma Chemical Co., St Louis, Mo.) as solid-phase capture system (32) and monoclonal and polyclonal antibodies to CTB and Ag I/II as probes (see below). Bovine serum albumin (BSA) (Sigma) was conjugated to Ag I/II with SPDP as described above.

To determine the amounts of free and bound Ag I/II and CTB present in the preparation, a sandwich ELISA protocol was used. Serial twofold dilutions of the conjugate and of purified CTB-SPDP and Ag I/II-SPDP derivatives were incubated in polystyrene wells that had previously been coated with G_{M1} ganglioside (31) and in wells coated with rabbit polyclonal IgG antibodies to Ag I/II; next, horseradish peroxidase-conjugated rabbit anti-Ag I/II or mouse monoclonal anti-CTB antibodies, appropriately diluted in phosphate-buffered saline containing 0.05% Tween 20, and enzyme substrate were applied sequentially to detect solid-phase bound CTB and Ag I/II. The amounts of free and bound Ag I/II and CTB were determined by reference to standard curves calibrated with known amounts of SPDP-derivatized antigens.

On average, the SPDP conjugation procedure described above yielded coupling efficiencies of approximately 80% for Ag I/II and 15% for CTB.

Immunizations. Female C57BL/6J mice, bred at the animal care facility of the Department of Medical Microbiology at the University of Göteborg (Göteborg, Sweden), were immunized at 8 to 14 weeks of age. Immunogens were diluted in 0.35 M $NaHCO_3$, and 0.5 ml of the solution containing the following amounts of immunogen was given by intragastric intubation to groups consisting of three to four mice each: 15 μg of Ag I/II-CTB conjugate with or without 5 μg of free CT as adjuvant; 15 μg of Ag I/II-CTB conjugate with 30 μg of free CTB; 15 μg of Ag I/II-BSA conjugate with 5 μg of CT; 150 μg of Ag I/II with or without 5 μg of CT. Immunizations were performed on days 0, 10, and 20. An additional group of mice received 6 doses of CT (5 μg each) given 10 days apart. Eight days after the last immunization, all mice were bled and then sacrificed by cervical dislocation.

Preparation of single-cell suspensions. Erythrocyte-free single-cell suspensions were obtained from spleen and mesenteric lymph nodes (MLN) as described previously (3). Single-cell suspensions from mouse salivary glands (SG) were obtained by enzymatic dispersion with trypsin. Submandibular glands were excised and freed from lymph nodes. The glands were then cut into fragments approximately 0.3 mm thick with a semi-automated tissue slicer (MacIlwain, Gomshall, United Kingdom). The fragments were rinsed twice with Hanks balanced salt solution (GIBCO, Glasgow, United Kingdom) and suspended in a siliconized flask with Hanks balanced salt solution containing 0.5% (wt/vol) trypsin (Difco Laboratories, Detroit, Mich.). After incubation for 30 min at 37°C with continuous shaking, single cells were collected by passing the contents of the digestion vessels through a nylon net. The procedure was repeated twice on the remaining fragments. Immediately after each digestion cycle, cells were pelleted by centrifugation (400 × *g*, 5 min) through a cushion of fetal bovine serum and suspended in Iscove medium (GIBCO) containing antibiotics and 5% fetal bovine serum. On average, the procedure described above yielded 2.1×10^6 mononuclear cells (range, 1.1×10^6 to 3.2×10^6) per pool of two submandibular SG with a viability (assessed by trypan blue dye exclusion) ranging from 60 to 85%.

Detection of antibody-producing cells. Spleen, MLN, and submandibular SG cell suspensions were assayed for numbers of specific antibody-secreting cells by enzyme-linked immunospot assay (ELISPOT) (3, 30) by using polystyrene dishes coated with *S. mutans* Ag I/II, CT, or BSA as described elsewhere (15, 29). After incubation of the cells in antigen-coated dishes for 4 h, zones of solid-phase bound immunoglobulin secreted from individual cells were visualized as dark brown spots by the stepwise addition of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin and agar containing chromogen substrate (3). Numbers of spot-forming cells (SFC) were determined on duplicate plates revealing 5 to 100 spots and adjusted to number of SFC per 10^7 mononuclear cells. Exposure of cell suspensions to cycloheximide (10^{-3} M) for 4 h at 37°C before and during plating abrogated spot formation, thus demonstrating that this assay detects cells synthesizing and actively secreting antibodies.

Cell cultures. Duplicate cultures consisting of 5×10^5 mononuclear cells from spleen, MLN, or SG in 0.2 ml of Iscove medium with antibiotics and 10% fetal bovine serum were incubated in flat-bottomed microwells (Nunc, Roskilde, Denmark) at 37°C in a humid atmosphere of 10% CO_2 in air. After 72 h, supernatants were harvested, clarified by centrifugation (600 × *g*, 10 min) and stored at −20°C until assayed.

ELISA. Serum samples from individual mice and culture supernatants from pooled (three animals) organs were assayed for levels of IgA, IgG, and IgM antibodies to Ag I/II and to CT by ELISA as previously described (29, 32). Briefly, serial twofold dilutions of samples were incubated in antigen-coated wells, and solid-phase bound antibodies were detected with affinity-purified goat anti-mouse IgA (Tago, Burlingame, Calif.), IgG (Organon Teknika, Malvern, Pa.), and IgM (Organon Teknika) antibodies followed by horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin (absorbed against mouse immunoglobulin) (Dakopatts,

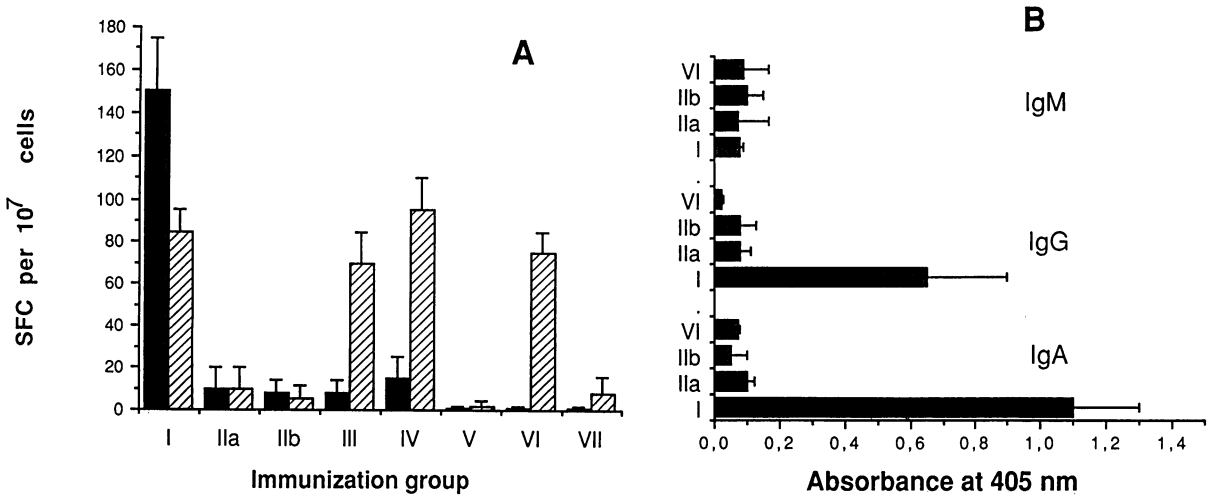

FIG. 1. Antibody responses in salivary glands from orally immunized C57BL/6J mice. Immunogens were given orally on days 0, 10, and 20 to groups of three to four mice: group I, 15 μg of Ag I/II-CTB conjugate with free CT; group II, as per group I but without free CT (IIa) and with free CTB (IIb); group III, 15 μg of Ag I/II-BSA conjugate with free CT; group IV, 150 μg of unconjugated Ag I/II with free CT; group V, as per group IV but without free CT; group VI, free CT alone given six times, 10 days apart; group VII, sham immunized. Eight days after the last immunization, single-cell suspensions from submandibular glands were assayed for numbers of cells producing antibodies to Ag I/II (■) or CT (▨) (A). Results are expressed as mean numbers of SFC from triplicate determinations plus or minus the standard error (vertical bars); SFC numbers in control (BSA-coated) dishes were always lower than $10/10^7$ cells. The isotypes of antibodies to *S. mutans* Ag I/II were determined by ELISA analyses of supernatants from cultured salivary gland cell suspensions (B). Results are expressed as mean ELISA absorbance (A_{414}) values of triplicate determinations plus or minus the standard error.

Copenhagen, Denmark). A_{414} was determined 30 min after addition of horseradish peroxidase chromogen substrate solution. The specificity of the antimouse reagents used was confirmed by ELISA by using purified mouse IgA and IgM paraproteins (a gift from Jiri Mestecky, University of Alabama, Birmingham) and purified mouse polyclonal IgG (KabiVitrum, Stockholm, Sweden) as solid-phase antigens. Serum titers were calculated as the highest sample dilution yielding an ELISA reading twice that of the control (no sample added). Such semiquantitative ELISA determinations allow comparisons between experimental groups only for levels of antibodies belonging to the same isotype.

RESULTS

Mucosal antibody-secreting cell responses to orally presented Ag I/II. Mice fed low doses (15 μg) of Ag I/II-CTB conjugate with free CT as adjuvant displayed high frequencies of Ag I/II-specific and CT-specific antibody-secreting cells (detected as SFC) in submandibular SG (Fig. 1A, group I). Even higher numbers of SFC were found in MLN (Table 1). Omission of CT adjuvant from the vaccine mixture prevented the generation of such responses (Fig. 1A, group IIa); free CTB failed to replace CT when coadministered with Ag I/II-CTB conjugate (Fig. 1A, group IIb). Equivalent doses of Ag I/II conjugated to BSA and given together with CT adjuvant failed to stimulate antibody responses to Ag I/II in SG, although substantial SFC responses to CT were recorded (Fig. 1A, group III); similar results were observed with MLN SFC responses (Table 1). Doses of Ag I/II as high as 150 μg, given alone or with CT adjuvant, were ineffective or at best poor in eliciting SFC responses to Ag I/II in SG (Fig. 1A, groups IV and V) as well as in MLN (Table 1). Likewise, CT given alone six times induced strong SFC responses to CT but not to Ag I/II in both SG (Fig. 1A, group VI) and MLN (Table 1). It is also noteworthy that the total numbers of lymphoid cells obtained from trypsin-dispersed

TABLE 1. Specific antibody-forming cells in MLN and spleens after oral immunization with CTB-conjugated Ag I/II

Group	Immunogen (dose [μg])[a]	CT (5 μg)	No. of SFC/10^7 cells[b]			
			Anti-Ag I/II		Anti-CT	
			MLN	Spleen	MLN	Spleen
I	Ag I/II-CTB (15)	+	1,170 ± 310	280 ± 55	2,400 ± 430	390 ± 60
IIa	Ag I/II-CTB (15)	−	<5	<5	ND	ND
IIb	Ag I/II-CTB (15) + CTB (30)	−	<5	<5	ND	ND
III	Ag I/II-BSA (15)	+	<5	<5	ND	ND
IV	Ag I/II (150)	+	60 ± 15	<5	2,030 ± 380	270 ± 40
V	Ag I/II (150)	−	<5	<5	<5	<5
VI		+[c]	<5	<5	3,500 ± 670	660 ± 110
VII		−	<5	<5	<5	<5

[a] Immunogens were given perorally on days 0, 10, and 20; antibody responses were determined on day 28.
[b] Data are expressed as mean number of SFC ± standard error of duplicate determinations. ND, Not determined.
[c] CT given perorally six times, 10 days apart.

TABLE 2. Serum antibody responses to *S. mutans* Ag I/II in C57BL/6J mice orally immunized with Ag I/II-CTB conjugate

Group	Immunogen (dose [μg])[a]	CT adjuvant (5 μg)	Serum anti-Ag I/II antibody titer[b]		
			IgA	IgG	IgM
I	Ag I/II-CTB (15)	+	3,840 ± 1,870	15,360 ± 7,220	<120
IIa	Ag I/II-CTB (15)	−	<120	<120	<120
IIb	Ag I/II-CTB (15) + free CTB (30)	−	<120	<120	<120
III	Ag I/II-BSA (15)	+	<120	<120	<120
IV	Ag I/II (150)	+	<120	480 ± 210	<120
V	Ag I/II (150)	−	<120	<120	<120
VI		+[c]	<120	<120	<120

[a] Immunogens were given perorally on days 0, 10, and 20; antibody responses were determined on day 28.
[b] Reciprocal of the serum dilution giving an ELISA absorbance value twice that of the control (no serum sample added); each value represents the mean serum antibody titer of three to four mice ± standard error.
[c] CT given perorally six times, 10 days apart.

SG were consistently higher in animals exposed to CT than in mice not treated with this adjuvant (data not shown). However, because of the limited numbers of submandibular SG lymphocytes available for performing class-specific ELISPOT assays, an alternative approach was used to determine the isotype distribution of Ag I/II-specific antibodies secreted by SG cells. Supernatants of short-term cultures of trypsin-digested submandibular SG tissues were analyzed by solid-phase ELISA. Both IgA and IgG, but not IgM, antibodies were detected (Fig. 1B). The same type of analyses performed on supernatants from MLN cell cultures yielded similar results with regard to isotype distribution of anti-Ag I/II antibodies (data not shown).

Splenic antibody responses to orally presented Ag I/II. Substantial numbers of cells secreting antibodies to Ag I/II were detected in the spleens of mice orally immunized with Ag I/II-CTB conjugate together with free CT (Table 1). Comparable responses to CT were observed in the spleens of these animals. Omission of free CT or replacement of CT by free CTB prevented the development of splenic antibody responses not only to Ag I/II but also to CT. Likewise, mice subjected to all other immunization protocols failed to develop detectable splenic antibody responses to Ag I/II.

Serum antibody responses to orally presented Ag I/II. ELISA analyses of the sera of orally immunized mice showed that high levels of circulating IgA and IgG antibodies to Ag I/II were developed in mice fed Ag I/II-CTB conjugate plus CT adjuvant but not in mice subjected to other immunization regimens (Table 2).

DISCUSSION

The results of these studies indicate that enteric immunization with microgram amounts of a protein antigen, *S. mutans* Ag I/II, can be effectively promoted by coupling the antigen to CTB. Similar and even greater doses of Ag I/II given alone or coupled to an irrelevant carrier (BSA) failed to elicit detectable antibody responses in any compartment examined. The mechanisms underlying processing of Ag I/II-CTB, or of intact CT, as well as presentation of these antigens to responsive lymphoid cells in the gut are as yet unknown, although binding of CTB to cell surface G_{M1} ganglioside is believed to be important (9). Extensive studies in rodents have revealed that CT possesses strong enteric adjuvant properties on gut immune responses to orally presented antigens (7, 15, 24). This activity is also demonstrated in the study presented here, in which a subclinical (5-μg) (9, 27) dose of free CT was necessary for the generation of optimal antibody responses to orally delivered Ag I/II-CTB in mice. In our experiments, purified CTB could not fulfill this role, indicating that the integrity of the native toxin is essential. However, as shown by clinical trials of oral cholera vaccine in humans, purified CTB devoid of any A subunit and therefore also of toxic activity is effective in inducing antibody responses as well as protection (10, 31). This suggests that in humans it may be possible to induce efficient mucosal immune responses to Ag I/II or other antigens coupled to CTB and given perorally without any additional adjuvant.

The demonstration of antibody-producing cells in salivary glands after enteric immunization with Ag I/II-CTB conjugate immunopotentiated by free CT lends further support to the concept that these glands belong to the common mucosal immune system (6, 12, 36), as has been demonstrated for other exocrine glands and mucosal tissues (reviewed in reference 20). In this regard, we have obtained preliminary evidence that oral immunization of human Swedish volunteers with CTB induces antibody-secreting cells in minor salivary glands (C. Czerkinsky, A.-M. Svennerholm, R. Jonsson, and J. Holmgren, manuscript in preparation). Similarly, the presence of even larger numbers of antibody-producing cells in MLN is consistent with the location of these lymph nodes on the lymphocyte migration pathway from gut-associated lymphoid tissue to the thoracic duct and circulation, and ultimately to the various submucosal tissues, where terminal differentiation into IgA-secreting plasma cells occurs (2, 13).

It is noteworthy that the immunization regimen described in the present studies resulted not only in mucosal responses but also in extramucosal IgA and IgG antibody responses. These were revealed by the induction of antibody-forming cells in the spleen and by the presence of high levels of IgA and IgG antibodies in serum. Similar effects have been observed after peroral immunization with CT in rodents (7, 15, 24, 27) and with a CTB-killed *Vibrio cholerae* whole-cell vaccine in humans (10, 31). Thus, it appears that the use of CT or CTB in a peroral vaccine evokes both mucosal and systemic immune responses.

In the particular context of immunization against the cariogenic bacterium *S. mutans*, partial protection against dental caries has been demonstrated in different animal models either by parenteral immunization, which elicits a systemic but not a mucosal antibody response (14), or by peroral immunization (without the benefit of CTB as carrier or CT as an adjuvant), which induces only a secretory antibody response (22). Thus, it may be anticipated that the induction of both arms of the immune response would be beneficial.

The theoretical reservation that repeated use of CTB (or of CT) as a carrier for oral delivery of different antigens might lead to decreased immune responsiveness due to an established gut antibody response to CTB does not seem to hold true, since multiple immunizations in both humans (31) and animals (15, 27) have proven to have booster effects with regard to gut mucosal antibody responses. Our data on salivary antibody responses in mice orally immunized with CT up to six times (Fig. 1A, group VI) are consistent with these observations.

In fact, this strategy of enhancing enteric immunization by means of CTB or other carrier proteins with known intestinal lectin-like binding properties (5) may have a more general application by providing secretory IgA antibodies that would act to inhibit colonization or invasion at mucosal surfaces and systemic antibodies that would act against an invasion that escaped the mucosal protective barrier. Furthermore, the enhanced immunogenicity of CTB-coupled antigens makes the peroral route comparable in dose effectiveness with conventional parenteral routes of vaccination.

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