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Induction of Mucosal Immunity by Intranasal Application of a Streptococcal Surface Protein Antigen with the Cholera Toxin B Subunit

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The level and distribution of isotype-specific antibodies in various secretions and of antibody-secreting cells in corresponding lymphoid organs and tissues were compared in mice immunized with *Streptococcus mutans* surface protein antigen I/II (AgI/II) conjugated to the cholera toxin B subunit (CTB), given intranasally (i.n.) or intragastrically (i.g.), with or without free cholera toxin (CT) as an adjuvant. Immunization i.n. induced stronger initial antibody responses to AgI/II in both serum and saliva than immunization i.g., but salivary immunoglobulin A (IgA)-specific antibody responses to immunization about 3 months later were not increased relative to total salivary IgA concentrations. Specific antibodies induced by i.n. immunization were as widely distributed in serum, saliva, tracheal wash, gut wash, and vaginal wash as those induced by i.g. immunization. Likewise, specific antibody-secreting cells were generated in the spleen, salivary glands, intestinal lamina propria, and mesenteric and cervical lymph nodes by either route of immunization. The strongest salivary IgA antibody response was induced by AgI/II-CTB conjugate given i.n., but the addition of CT did not further enhance it. However, free CTB could effectively replace CT as an adjuvant in i.n. immunization with unconjugated AgI/II. Booster i.n. immunization with AgI/II plus either free CT or CTB induced stronger recall serum antibody responses than conjugated AgI/II-CTB with or without CT as an adjuvant. Therefore, i.n. immunization with a protein antigen and free or coupled CTB is an effective means of generating IgA antibody responses expressed at several mucosal sites where protective immunity may be beneficial.

The surface fibrillar protein antigen I/II (AgI/II) of *Streptococcus mutans* is a strongly immunogenic protein when given parenterally and is considered to be a promising candidate for a vaccine against dental caries (31). For this purpose, it is desirable to elicit a salivary immunoglobulin A (IgA) antibody response, which is most effectively achieved by stimulating the common mucosal immune system (29). According to this concept, antigens presented at central inductive sites, such as the gut-associated lymphoid tissues (GALT), induce the clonal expansion of specific IgA-committed B cells and appropriate T cells, which then emigrate and ultimately settle in remote effector sites of mucosal immunity, including the salivary glands. Here, terminal differentiation of the B lymphoblasts into polymeric IgA-secreting plasma cells occurs under the control of stimulated T cells, and the product, secretory IgA, is finally released into the external secretion. Like other soluble proteins, AgI/II given alone perorally does not readily induce mucosal or systemic immune responses (8), probably because it is readily digested and not efficiently taken up by the GALT. However, we have demonstrated that in mice, intragastric (i.g.) administration of microgram amounts of AgI/II conjugated to cholera toxin B subunit (CTB) could induce both serum IgG and IgA antibodies and salivary IgA antibodies to AgI/II, which lasted for at least 6 months (8, 32). Cholera toxin (CT) is exceptionally effective as an enteric immunogen and adjuvant in experimental animals (6, 7, 11, 20, 24). Antigens given perorally together with CT induce IgA responses in mucosae and IgG and IgA antibodies in plasma to both components. We found that CT was necessary as an adjuvant for AgI/II-CTB conjugates given i.g. in microgram doses and that conjugation of AgI/II to CTB was essential for these doses to induce effective responses to AgI/II in both mucosal and systemic sites (8, 32). However, the responses varied among animals, and some animals failed to respond even after three doses of 150 μg of AgI/II-CTB conjugate. Furthermore, it is not feasible to use CT in humans as it is highly toxic, and the ability of nontoxic CTB to act as a mucosal adjuvant has been debated: various results have been obtained, depending upon the antigen, dose, route, and species involved (6, 8, 28, 32, 34, 35, 40).

Intranasal (i.n.) immunization has been successfully used to induce antibodies to a variety of antigens administered with CTB or CT (1, 30, 34, 35). We have therefore evaluated i.n. immunization with AgI/II conjugated to CTB and mixed with CT as an adjuvant in comparison with i.g. immunization, particularly to determine whether salivary IgA antibody responses to a bacterial protein antigen can be more consistently obtained. Because we previously found that, with i.g. immunization, it was necessary to couple AgI/II to CTB and give free CT as an adjuvant, whereas different results have been obtained by i.n. immunization with other antigens, we investigated the requirement for coupling with CTB and for CT as an adjuvant to generate antibodies to AgI/II given i.n. and their effects on the duration and recall of the responses. Furthermore, the distributions of antibody responses induced by i.g. and i.n. immunization among different mucosal effector sites have been compared to determine whether both routes elicit similar responses in various components of the common mucosal immune system.

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MATERIALS AND METHODS

Antigens. *S. mutans* AgI/II was prepared and conjugated to CTB (List Biological Laboratories, Inc., Campbell, Calif.) as previously described (32), by using *N*-succinimidyl-(3-[2-pyridyl]-dithio)propionate as the coupling agent and equimolar amounts of AgI/II and CTB. The conjugate was tested by gel permeation chromatography on a Superose 6 column (Pharmacia LKB Biotechnology, Piscataway, N.J.), and by enzyme-linked immunosorbent assay (ELISA) on plates coated with G_{M1} ganglioside and developed with peroxidase-conjugated antibody to AgI/II, as previously described (32). All batches showed similar abilities to bind to G_{M1} ganglioside and retained similar AgI/II antigenicities. The concentration of the conjugate was determined by A_{280}, by using extinction coefficients determined for both CTB and AgI/II, and weighted for the proportions present in the conjugate. CT was also obtained from List Biological Laboratories.

Animals and immunization. Adult female BALB/c mice, 18 to 20 weeks old, from a pathogen-free colony, were used for all experiments. Groups of five mice were immunized three times at 10-day intervals either i.g. by gastric intubation with the immunogen dose in 0.25 ml of 0.1 M $NaHCO_3$ (32) or i.n. with the dose in 10 to 20 μl of phosphate-buffered saline (PBS), which was introduced slowly (5 μl at a time) into the external nares by means of a micropipettor. In some experiments, mice received a single booster immunization on day 118 with the same dose and in the same form and route as used previously.

Serum and saliva samples were collected on day 0 and, in some experiments, sequentially 7 days after each immunization and at additional intervals after the third dose, for assay of antibodies by ELISA (see below). In other experiments, mice were killed 7 days after the third immunization, and serum, saliva, tracheal wash, gut wash, and vaginal wash samples were collected for analysis of antibodies. Spleen, submandibular and sublingual salivary glands, superficial cervical lymph nodes (CLN), small intestinal lamina propria (LP), and mesenteric lymph nodes (MLN) were excised and pooled according to experimental groups to prepare suspensions of mononuclear cells (MNC) for analysis of antibody-secreting cells by the enzyme-linked immunospot (ELISPOT) assay (see below).

Cell preparations. Spleens and lymph nodes were teased apart with needles. Single MNC suspensions were obtained by vigorous pipetting, and tissue debris was removed by filtering through nylon mesh. The erythrocytes were lysed, and nonviable cells were removed by centrifugation on Histopaque 1083 (Sigma Diagnostics, St. Louis, Mo.).

The LP MNC were obtained by the method previously described (21, 42). Peyer's patches were excised from the small intestine, and the epithelium was removed by shaking in Ca^{2+}- and Mg^{2+}-free Hanks' solution with 5 mM EDTA. The tissue was digested with three cycles of collagenase, and the cells recovered from each cycle were washed and pooled. Tissue debris was removed by centrifugation through 40% Percoll (Pharmacia).

Salivary gland MNC were prepared by a modification of the previously described method (32), in which digestion with two 45-min cycles of collagenase (type IV, 2.5 U/mg of tissue; Sigma) and hyaluronidase (type IV, 14 U/mg of tissue; Sigma) in 10 ml of Iscove's medium with 5% fetal calf serum (FCS) was used (3). The cells collected from each digestion were further treated with Dispase (Boehringer Mannheim Corp., Indianapolis, Ind.) (0.05 mg/ml) for 10 min on ice and washed with 10% FCS in Iscove's medium.

The cells from different organs were resuspended in 10% FCS in Iscove's medium, counted in a hemacytometer with trypan blue to estimate viability, and used for the ELISPOT assay.

ELISPOT assay. Total Ig- and specific antibody-secreting cells were enumerated by ELISPOT assay on 96-well membrane-based plates (Millipore Inc., Bedford, Mass.) coated with anti-Ig or with AgI/II or CT, as described previously (32). Suspensions of viable cells at appropriate concentrations were incubated in duplicate wells for 3 to 4 h, and the plates were developed with isotype-specific goat anti-mouse Ig-peroxidase conjugates (Southern Biotechnology Associates, Birmingham, Ala.). The spots formed with 3-amino-9-ethylcarbazole plus H_2O_2 were counted under a stereo microscope and spot-forming cells (SFC) were expressed relative to 10^6 cells plated.

Collection of fluids. Serum was obtained from tail vein blood or from blood collected from the subclavian veins when mice were sacrificed. Saliva was collected as described previously (32) after stimulation of secretion with carbachol and stored at −20°C.

Gut wash was obtained by flushing the small intestine with 2 ml of Dulbecco's PBS into 3 ml of 50 mM EDTA containing 0.1 mg of soybean trypsin inhibitor (Sigma) per ml, made up to 6 ml, decanted from solid material, vortexed, and centrifuged for 10 min at 650 × *g*. The top 5 ml was removed, mixed with 50 μl of 100 mM phenylmethylsulfonyl fluoride in 95% ethanol, and centrifuged at 27,000 × *g* for 20 min at 4°C. The top 4 ml was taken, mixed with a further 40 μl of 100 mM phenylmethylsulfonyl fluoride and 40 μl of 1% NaN_3. After standing for 15 min, 200 μl of FCS was added, and the fluid was stored at −20°C (13).

Tracheal wash was obtained by opening the exposed trachea to insert a polypropylene cannula, which was tied in place and connected to a syringe. The thorax was opened, avoiding damage to the lungs, and 1 ml of Dulbecco's PBS was injected into the trachea and withdrawn three times. The fluid was stored at −20°C and centrifuged before being assayed.

Vaginal wash was obtained by introducing 50 μl of Dulbecco's PBS with a 200-μl pipettor and withdrawing it five times. The fluid was stored at −20°C and centrifuged before being assayed.

ELISA. Isotype-specific antibodies and total Ig isotype concentrations were determined by ELISA on 96-well plates coated with AgI/II, CT, or isotype-specific anti-Ig, as described previously (32). The plates were developed with goat anti-mouse IgM-, IgG-, or IgA-peroxidase conjugates (Southern Biotechnology Associates) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate with H_2O_2. Unknowns were calculated by interpolation on calibration curves constructed by a computer program based on four parameter logistic algorithms, as previously described (41). Antibody levels in secretions were expressed relative to corresponding total Ig isotype concentrations, to adjust for fluctuations in secretion flow rate or for variable dilution effects in washing procedures.

Statistical methods. Results of i.n. and i.g. immunizations were evaluated by Student's *t* test (unpaired samples), and responses to initial and booster immunizations were evaluated by Student's *t* test for paired samples. Multiple group comparisons of different i.n. immunization regimens were evaluated by one-factor analysis of variance. All calculations were made by means of the StatView program (Abacus Concepts, Berkeley, Calif.) with a Macintosh SE computer.

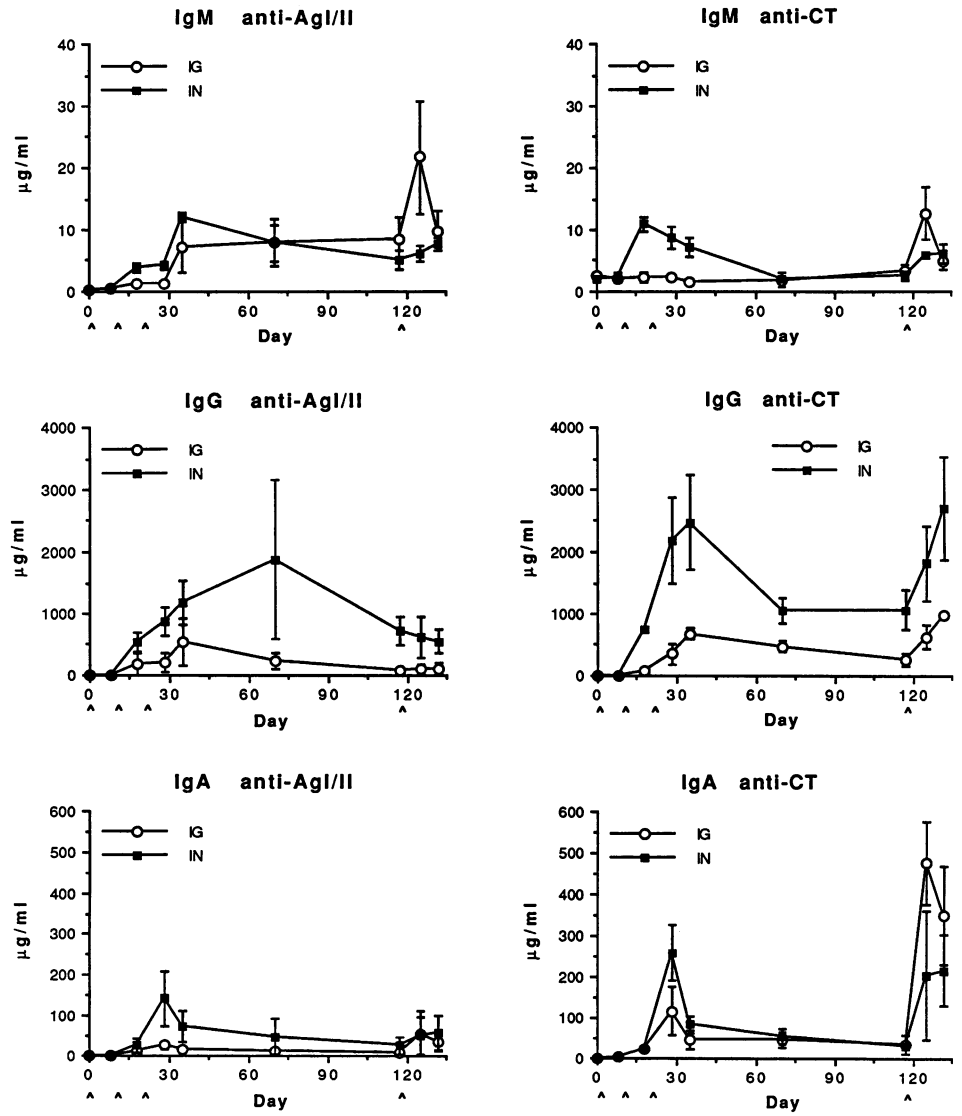

FIG. 1. Time course of serum antibody responses to AgI/II and CT in mice immunized i.g. or i.n. with AgI/II-CTB conjugate and free CT as adjuvant on days 1, 11, 21, and 118 (Λ). Results are the means ± standard deviations for five mice per group. It should be noted that the mean and standard deviation of the anti-AgI/II IgG response on day 70 to i.n. immunization were elevated because one sample (from an animal that gave consistently strong responses) had an exceptionally high value; otherwise, the peak response occurred on day 35.

RESULTS

Comparison of i.n. and i.g. routes of immunization. To compare the antibody responses in serum and saliva samples of mice immunized either i.g. or i.n., two groups of mice were immunized with three doses of 15 µg of AgI/II-CTB conjugate plus 5 µg of CT by either route at 10-day intervals. Serum anti-AgI/II and anti-CT antibody levels in i.n. immunized mice were raised higher than those in i.g. immunized mice after the initial immunization (Fig. 1). The peak levels of serum specific IgG (on day 35) and IgA (on day 28) antibodies were significantly higher (P, <0.05) in the i.n. immunized group than in the i.g. immunized group. Peak levels of IgM anti-AgI/II antibodies on day 35 were not significantly different (P, 0.054), but IgM anti-CT antibodies peaked on day 17 in the i.n. immunized group at a significantly higher level (P, <0.05) than in the i.g. immunized group.

The proportions of salivary IgA antibodies against AgI/II and CT relative to the total IgA concentration, as well as the actual antibody concentrations in saliva, were elevated in both i.n. and i.g. immunized groups (Fig. 2). The i.n. immunized mice showed significantly higher peak levels (P, <0.005, on day 35) of anti-AgI/II and anti-CT IgA than i.g. immunized mice.

After a single booster immunization about 3 months later, the recall responses were more diverse than after the initial immunization. Only the i.g. immunized group showed substantial serum IgM antibody responses 7 days after the booster immunization (Fig. 1), and these were greater than those seen after initial immunization (P, <0.005). There were no serum recall IgG responses to AgI/II in either immunized group, although the serum anti-AgI/II IgG remained significantly higher in the i.n. immunized group (P, <0.005), indicating that no systemic memory response was

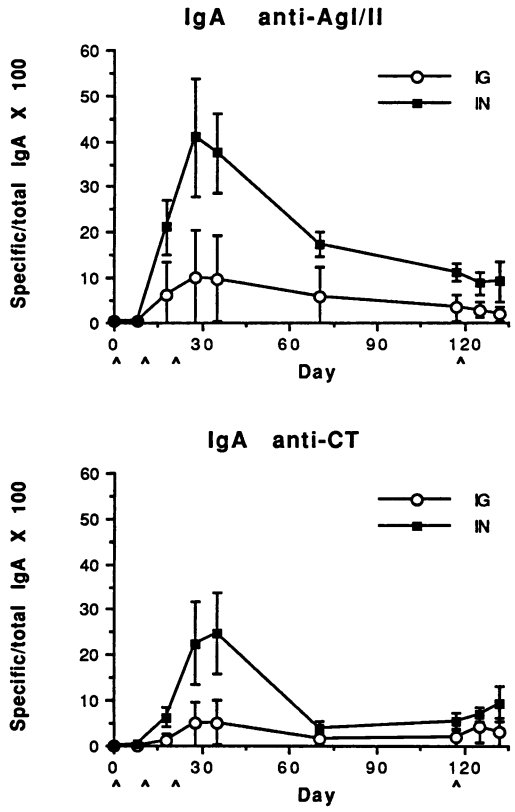

FIG. 2. Time course of salivary IgA antibody responses to AgI/II and CT in mice immunized i.g. or i.n. with AgI/II-CTB conjugate and free CT as adjuvant on days 1, 11, 21, and 118 (∧). Results are the means ± standard deviations of specific antibody levels relative to the total IgA concentration for five mice per group.

obtained against AgI/II. In contrast, serum anti-CT IgG was elevated in both the i.n. and i.g. immunized groups upon booster immunization, and as in the initial responses, the IgG anti-CT antibody level was significantly higher in the i.n. immunized group 14 days after boosting (P, <0.002). Serum anti-AgI/II and anti-CT IgA showed patterns similar to those of IgM after booster immunization. The anti-AgI/II IgA level was not significantly different in the two groups and not higher than that after initial immunization. However, in the i.g. immunized group, serum anti-CT IgA level was dramatically elevated over that during the initial response (P, <0.005) and was significantly higher than that in the i.n. boosted mice (P, <0.05), indicating that i.g. immunization was more capable of inducing memory responses to CT than i.n. immunization.

Salivary IgA antibody concentrations relative to total IgA concentrations did not increase after booster immunization on day 118 by either route (Fig. 2). However, total salivary IgA concentrations as well as specific IgA antibody levels were concordantly increased in both groups (data not shown), as observed previously (32).

Antibody responses in different secretions after i.n. and i.g. immunization. To compare the distribution of antibody responses after i.n. and i.g. immunization, groups of mice were immunized with AgI/II-CTB and CT according to the previous initial schedules, except that the i.g. dose of AgI/II-CTB conjugate was increased to 30 μg in an attempt to make the overall level of responses similar in both groups. Various secretion samples were collected 7 days after the third dose for assay of antibodies, and the associated tissues were excised and processed to obtain MNC for ELISPOT assays.

Salivary IgA antibodies to both AgI/II and CT after i.g. immunization were enhanced by the increased dose of immunogen (Table 1), compared with the responses illustrated in Fig. 2. However, as the concentration of total salivary IgA was greater in the i.n. immunized animals, the actual IgA antibody levels to both antigens appeared to be higher in this group. A marked increase in total salivary IgA

TABLE 1. Total Ig and specific antibody levels in the secretions of mice immunized i.g. or i.n. with AgI/II-CTB conjugate plus free CT

Sample source	Immunization route	Mean (× SD) concn (μg/ml) of [a]:			% Mean concn (÷ SD) of [b]:					
					Anti-AgI/II			Anti-CT		
		IgM	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA
Saliva	None (control)	ND	ND	3.43 (1.47)	ND	ND	0	ND	ND	0
	i.g.	ND	ND	3.24 (1.82)	ND	ND	40.05 (2.51)	ND	ND	15.31 (3.23)
	i.n.	ND	ND	24.40 (2.02)	ND	ND	30.21 (2.08)	ND	ND	36.88 (2.35)
Gut wash	None (control)	0.18 (2.02)	2.24 (1.93)	113.39 (1.66)	0	0	0	0	0	0
	i.g.	0.10 (1.07)	1.90 (1.35)	94.93 (1.30)	0	12.26 (1.71)	1.77 (1.97)	0	4.08 (1.18)	15.02 (1.45)
	i.n.	0.07 (1.42)	3.08 (1.61)	89.18 (1.25)	0	25.86 (1.16)	3.66 (1.40)	0	15.30 (1.43)	3.99 (1.85)
Tracheal wash	None (control)	0.07 (1.51)	5.59 (1.78)	1.33 (1.32)	0	0	0	0	0	0
	i.g.	0.05 (2.79)	3.55 (2.23)	1.00 (2.84)	0	12.96 (2.72)	8.93 (6.95)	0	7.46 (1.17)	47.43 (2.39)
	i.n.	0.07 (1.22)	10.65 (1.22)	2.34 (1.12)	0	15.39 (1.48)	22.29 (1.26)	0	30.57 (1.20)	52.85 (1.47)
Vaginal wash	None (control)	ND	ND	12.52 (2.06)	ND	ND	0.14 (1.59)	ND	ND	0.27 (3.04)
	i.g.	ND	ND	38.22 (1.67)	ND	ND	8.76 (2.20)	ND	ND	7.29 (2.46)
	i.n.	ND	ND	75.60 (2.20)	ND	ND	22.87 (1.90)	ND	ND	14.71 (4.89)

[a] Geometric mean ×/÷ standard deviation; individual zero values in saliva and vaginal wash were taken as 0.01. ND, not determined.
[b] Specific antibody concentration/total Ig isotype concentration × 100. 0, virtually undetectable concentration in all samples.

TABLE 2. Cells secreting antibodies to AgI/II and CT in various organs of mice immunized i.g. or i.n. with AgI/II-CTB conjugate plus free CT[a]

Cell source	Immunization route	No. of SFC/10^6 MNC secreting:					
		Anti-AgI/II			Anti-CT		
		IgM	IgG	IgA	IgM	IgG	IgA
Spleen	i.g.	63	38	85	28	5	61
	i.n.	350	100	293	50	58	193
SG	i.g.	ND	ND	2,316	ND	ND	383
	i.n.	ND	ND	10,100	ND	ND	7,800
LP	i.g.	470	53	3,000	20	105	38,500
	i.n.	135	70	1,095	75	50	3,670
CLN	i.g.	15	20	20	5	0	20
	i.n.	195	300	220	125	295	265
MLN	i.g.	5	5	0	0	10	65
	i.n.	0	0	10	0	5	0

[a] Unimmunized control mice had <5 SFC/10^6 cells in all cases except for anti-CT IgA in salivary glands (SG) (185 SFC/10^6 cells) and in LP (235 SFC/10^6 cells). ND, not determined.

levels during peroral immunization with AgI/II-CTB conjugates had been noted previously (32), but it is not clear why only the i.n. immunized animals showed this effect in the present experiment.

IgG and IgA antibodies to AgI/II and CT were detectable in gut wash samples from both immunized groups (Table 1). IgA was the predominant isotype overall, although the proportion of specific antibody relative to total IgG was higher in some instances. Immunization i.n. resulted in greater IgG antibody responses to both antigens and greater anti-AgI/II IgA response, but anti-CT IgA response was substantially higher after i.g. immunization. It is possible that IgG antibodies detected in gut wash reflect either minor contamination with blood during collection or transudation from plasma in vivo, as the ratios of IgA to IgG antibody-secreting cells in the LP were higher (see below) than those of IgA to IgG antibodies in gut wash. However, IgM concentrations were low, and specific IgM antibodies were virtually undetectable in gut wash.

In tracheal wash, both i.n. and i.g. immunization generated anti-AgI/II IgG and anti-CT IgA antibodies to similar extents, but i.n. immunization induced greater IgG anti-CT and IgA anti-AgI/II responses (Table 1). Although IgG was the predominant isotype, the ratios of specific IgA to IgG antibodies were greater than the corresponding ratios of total IgA to IgG, implying a relative enrichment of specific IgA antibodies.

The yield of vaginal wash was sufficient for only the determination of IgA responses (Table 1). Both routes of immunization induced IgA antibodies to AgI/II and CT, but as specific antibody levels and the concentrations of total IgA were greater in the i.n. immunized animals, the response appeared to be higher in this group.

Distribution of antibody-secreting cells after i.n. and i.g. immunization. Immunization by the i.n. route induced larger numbers of AgI/II- and CT-specific SFC of all isotypes in the spleen and of IgA isotype in the salivary glands than i.g. immunization (Table 2). In contrast, i.g. immunization induced stronger IgA SFC responses to AgI/II and especially to CT in LP. When the CLN that drain salivary glands were examined, again substantially larger numbers of AgI/II- and CT-specific SFC of all isotypes were found in i.n. immunized mice than in i.g. immunized mice (Table 2). In MLN, few specific SFC were found, the largest number being cells secreting anti-CT IgA in mice immunized i.g. (Table 2).

Requirement for coupling AgI/II to CTB and for CT as an adjuvant in i.n. immunization. Previous experiments (8, 32) demonstrated that responses to i.g. immunization with small doses of AgI/II and CTB depended upon chemical coupling of AgI/II and CTB and the coadministration of a small adjuvant dose of intact CT. To determine whether the same requirements applied also to i.n. immunization, groups of mice were immunized i.n. with 15 μg of AgI/II-CTB conjugate with or without 5 μg of free CT or with 10 μg of free AgI/II alone or mixed with 5 μg of either free CTB or free CT. The mice were immunized three times at 10-day intervals. IgA antibody responses were determined in samples of saliva taken 7 days after each immunization and 14 days after the third dose (Fig. 3). The strongest anti-AgI/II IgA responses were given by mice immunized with AgI/II-CTB conjugate, with or without free CT, and no difference between these two groups was discernible. Lower, but still strong, responses were also shown in mice given AgI/II mixed with either free CTB or free CT, and again there was no difference in response between these two groups. Mice immunized with AgI/II alone gave clearly detectable, though substantially lower, salivary IgA responses. One-way analysis of variance of the responses at day 35 indicated significant differences between i.n. immunization with AgI/II-CTB conjugate and AgI/II plus free CTB, AgI/II-CTB conjugate plus free CT and AgI/II plus free CT, and AgI/II alone and all other combinations. Thus, free CT had no enhancing effect for AgI/II-CTB conjugates given i.n., but either free CT or free CTB could enhance responses to free AgI/II. However, immunization with AgI/II-CTB conjugate i.n. gave the strongest salivary IgA responses.

Salivary IgA anti-CT responses were highest in animals receiving free CT, irrespective of other components, and lower in mice given free or combined CTB without free CT (Fig. 3). Mice immunized with AgI/II alone did not develop antibodies to CT.

There were no statistically significant differences in serum IgG and IgA antibody responses to AgI/II between groups immunized with AgI/II and free or coupled CTB or CT as

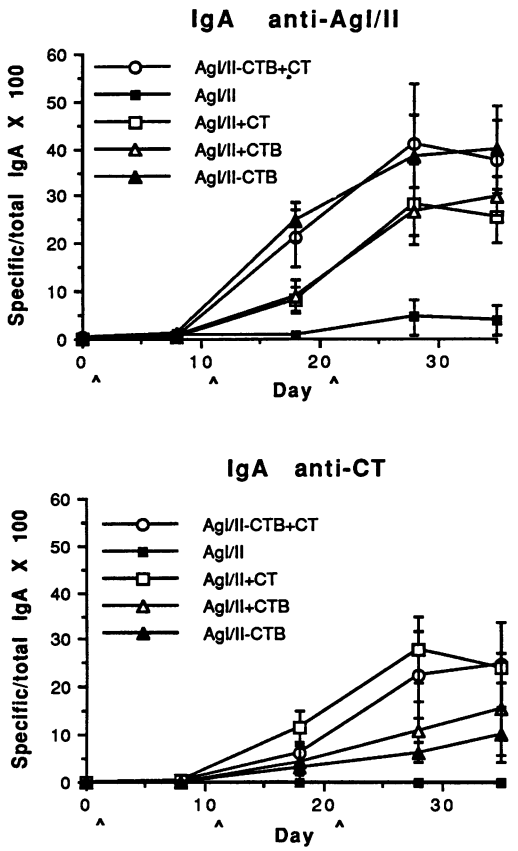

FIG. 3. Time course of salivary IgA antibody responses to AgI/II and CT in mice immunized i.n. with AgI/II-CTB conjugate with or without free CT or with free AgI/II alone or mixed with free CTB or free CT, on days 1, 11, and 21 (^). Results are the means ± standard deviations of specific antibody levels relative to the total IgA concentration for five mice per group.

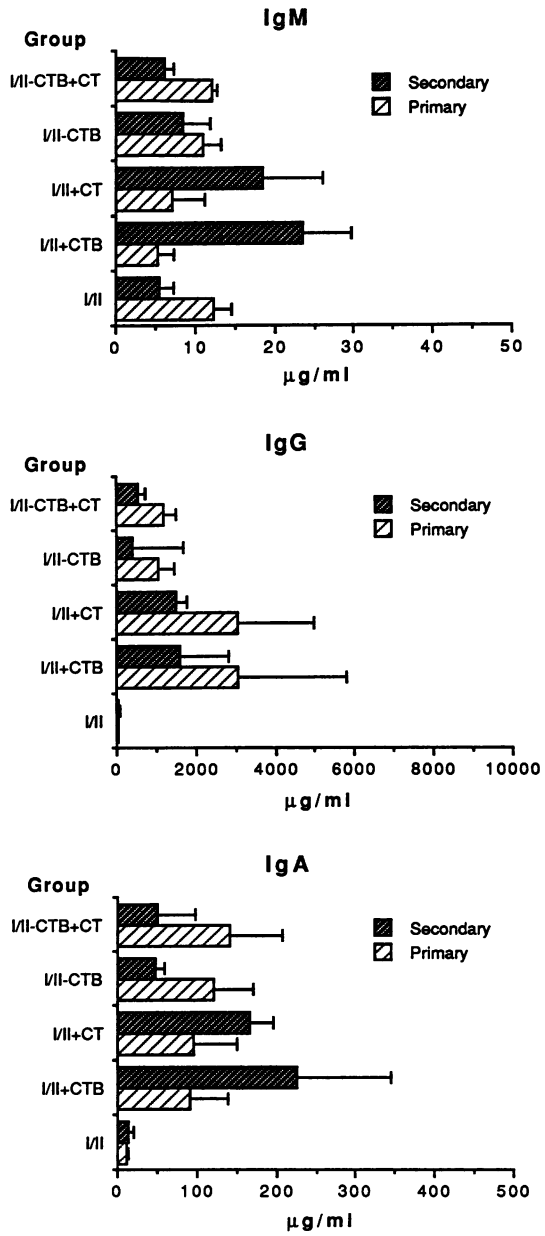

FIG. 4. Serum antibody responses to AgI/II in mice immunized i.n. with AgI/II-CTB conjugate with or without free CT or with free AgI/II alone or mixed with free CTB or free CT, on days 1, 11, 21, and 118. Results shown are the means ± standard deviations of peak primary (day 28 for IgA; day 35 for IgM and IgG) or peak secondary (day 125 for IgM and IgA; day 132 for IgG) responses for five mice per group.

adjuvant, although the mean levels of anti-AgI/II IgG tended to be higher on day 35 in mice given AgI/II plus free CT or free CTB as adjuvant (Fig. 4). As expected, the serum IgG and IgA responses to CT were significantly stronger in mice given free CT irrespective of other immunogen components (data not shown).

Anamnestic responses to AgI/II and CT in i.n. immunized mice. Previous experiments (32) have shown that i.g. immunization with AgI/II-CTB conjugate and free CT did not establish significant capacity to mount anamnestic responses in salivary IgA antibodies to AgI/II in response to a single subsequent immunization. A similar result was found after i.n. immunization with AgI/II-CTB conjugate and free CT (see above). However, i.g. immunization did prime mice for substantial recall serum antibody responses to both AgI/II and CT (32). To determine whether the different regimens of i.n. immunization varied in their abilities to induce anamnestic antibody responses, the same animals were given a single booster immunization of the same material and dose about 3 months later when responses to the initial immunizations had declined, and serum and saliva samples were collected 7 and 14 days later.

Peak serum IgM and IgA recall responses, measured at 7 days after booster immunization, to AgI/II were significantly greater (P, <0.01 for IgM; P, <0.05 for IgA; paired Student's t test) than initial responses only in groups given AgI/II plus free CT or free CTB (Fig. 4). In contrast, mice given AgI/II-CTB conjugate, with or without free CT, showed lower peak recall IgM, IgG (at 14 days), and IgA responses (P, <0.05 for all isotypes) compared with those obtained after initial immunization (Fig. 4), and mice immunized with AgI/II plus free CT or free CTB gave lower recall IgG responses, though these were not statistically significant (Fig. 4). Thus, it appears that enhanced anamnestic serum IgG antibody responses to AgI/II were not elicited by i.n. immunization. There were no specific increases in salivary

IgA antibodies relative to total IgA concentrations after i.n. booster immunization, but total salivary IgA levels were increased in all groups (data not shown), as observed previously (see above) and after i.g. booster immunization (32).

DISCUSSION

Parallel experiments showed clearly that after three similar doses of immunization with AgI/II-CTB conjugate and free CT, the responses in both saliva and serum were significantly stronger in i.n. immunized mice than in i.g. immunized mice. Several factors may contribute to these findings. As intranasal cavities contain less proteolytic activity than the intestinal lumen in which protein antigens can be extensively degraded, similar small amounts of antigen may be more effectively delivered to stimulate the mucosal immune system by the i.n. route than by the i.g. route. Of greater immunological interest, however, is the fact that the nasal passages of rats and mice contain organized lymphoid tissue that is considered to be the equivalent of Waldeyer's ring in humans (19). This nasal-associated lymphoid tissue (NALT) consists of follicles covered by domes of specialized epithelium resembling the M cells of intestinal Peyer's patches, and the underlying lymphoid cells include $CD4^+$ and $CD8^+$ T cells, with a preponderance of the former. Thus, it is possible that i.n. administered antigens were taken up by these tissues and induced a mucosal immune response in a fashion analogous to stimulation of GALT. However, it appears that NALT is more adept at taking up particulate rather than soluble antigens which may be more readily adsorbed through the nasal mucosa (19). Furthermore, the lymphoid drainage of NALT is mainly to the posterior CLN directly, whereas the nasal mucosa drains predominantly first to the superficial CLN (18, 36). Our observations of antibody-secreting cells in the superficial CLN therefore support the notion of immunization preferentially through the nasal mucosa. In humans, it has been reported that $CD4^+$ T cells outnumber $CD8^+$ T cells among intraepithelial and submucosal lymphocytes of nasal mucosae, unlike in the intestinal mucosa where $CD8^+$ cells predominate (4). If $CD4^+$ T cells also predominate in the nasal mucosa of mice, this may help to explain the effectiveness of i.n. immunization with soluble antigens that we observed. Indeed, in our experiments with i.n. immunized mice, three doses of 10 μg of free AgI/II alone could induce serum IgG and IgA responses as well as salivary IgA antibody responses. This amount of AgI/II completely failed to induce responses when administered i.g. in mice, even when free CTB and free CT were included as an adjuvant (8, 32).

In another experiment, in which mice were given 30 μg of AgI/II-CTB conjugate and 5 μg of CT intraorally, the responses in serum and saliva samples were similar to those for i.g. immunized mice (33) but much lower than those obtained for i.n. immunized mice. This eliminated the possibility that strong immune responses induced by antigen administered i.n. might be due to antigen entering the mouth and stimulating oral lymphoid tissue reported to occur in minor salivary glands (3, 10) or being swallowed and stimulating the GALT. It also seems unlikely that much antigen administered i.n. entered the trachea and stimulated the bronchus-associated lymphoid tissue, because mucociliary flow is in the opposite direction. However, this possibility cannot be entirely discounted at present.

To determine the distribution of antibody response in mucosal and systemic sites after i.n. immunization, we compared antibodies in saliva, tracheal wash, gut wash, and vaginal wash, and antibody-secreting cells in spleen, salivary glands, LP, and their draining lymph nodes in i.n. immunized mice with those in i.g. immunized mice. Immunization i.n. induced antibody responses in secretions and antibody-secreting cells in various organs as widely distributed as did immunization i.g. The route of cell migration after i.n. immunization is probably through superficial and posterior CLN (18, 36), and we have found that, 4 days after the last i.n. immunization (possibly earlier), antibody-secreting cells appeared in superficial CLN as well as in the posterior CLN (unpublished observations). In particular, the superficial CLN were substantially enlarged compared with those in unimmunized mice. It is therefore likely that soluble antigens applied to the nasal cavities were absorbed through epithelial cells (19) and stimulated the intraepithelial and submucosal lymphoid cells, which then drain to local lymph nodes and migrate to remote mucosal effector sites. The finding of few specific antibody-secreting cells in MLN is consistent with our previous observations (32) that these cells attain peak numbers soon after immunization and rapidly decline for 3 to 7 days thereafter, presumably as they migrate elsewhere in the common mucosal immune system.

Immunization i.n. with AgI/II-CTB conjugate induced very strong salivary IgA antibody responses, but the addition of CT as an adjuvant had no further enhancing effect. On the other hand, free CT or CTB exerted an adjuvant effect when administered i.n. with unconjugated AgI/II. These results were different from those of our previous i.g. immunization experiments (32), in which we found that coupling of AgI/II to CTB and the addition of CT as an adjuvant were necessary to generate effective mucosal responses. The present results therefore show that the effects of CTB as a carrier and of CT as an adjuvant are different in i.n. and i.g. routes of immunization. Although CTB alone had no adjuvant activity in i.g. immunization in some studies (8, 16, 24, 32), it was apparently successful in others (6, 40), especially when coupled to the antigen (2, 9, 28). Furthermore, the requirement for CTB conjugation in i.g. immunization seems to depend on the dose of protein antigen administered: admixture of free CT, with or without free CTB, has an adjuvant effect with multimilligram amounts of protein antigens (12, 38, 40) but not with microgram amounts (8, 24, 32).

Several mechanisms may contribute to the adjuvant activity of CT and CTB by the i.n. route. Both CT and CTB are reported to enhance penetration of antigens through the epithelia (15, 25). The binding of CT to M cells of intestinal Peyer's patches (37) suggests that the uptake of other proteins would be promoted by conjugation to CTB, and if this occurs in NALT, then it might explain the enhanced salivary immune response to AgI/II-CTB. In addition, both CT and CTB have been shown to have various stimulating effects on immunocompetent cells, including antigen-presenting cells (5, 17, 22), $CD4^+$ T cells (7, 27, 39), and B cells (14, 26).

A remarkable effect that we have observed previously in perorally immunized mice (32) is the increase in total salivary IgA levels during i.n. immunization. The increase appears to coincide with immunization with CT and CTB (32), irrespective of whether AgI/II is administered, but it has not yet been rigorously investigated and the mechanism responsible is not obvious. However, because total salivary IgA levels can increase in this manner, it is important that salivary IgA antibodies be related to total IgA concentration, in order to differentiate a specific immune response from a nonspecific elevation.

Although i.n. immunization induced stronger initial responses than i.g. immunization, specific salivary IgA antibody responses to recall immunization by either route were poor, although booster i.g. immunization with AgI/II-CTB and CT induced strong serum IgA responses, especially to CT (32). In contrast, booster i.n. immunization with free AgI/II plus either free CT or free CTB, but not with AgI/II-CTB conjugate, did result in enhanced serum IgM and IgA antibody responses. The mechanisms responsible for these results are not clear. If the coupling of AgI/II to CTB promotes uptake by M cells, germinal center development in GALT may result in dissemination of a strong booster response to i.g. immunization that is preferentially expressed in serum, or possibly in the intestinal LP (23). Upon i.n. immunization, however, similar targeting of AgI/II-CTB conjugates to NALT, which has few germinal centers compared with GALT (19), might be ineffective for inducing a booster response. In contrast, the absorption of free AgI/II by nasal mucosae, enhanced by the presence of free CT or CTB, might generate booster responses by a different mechanism. In neither case, however, is it clear why the booster responses should be expressed more strongly in serum than in saliva.

Our results suggest a promising way to develop a nasally applied vaccine for inducing secretory IgA antibodies in saliva and other secretions, by using free or coupled CTB as an enhancing agent that may be acceptable in humans. However, the desirable goal of providing long-term anamnestic immunity, as well as short-term responses, may require a combination of routes or both CTB-conjugated and unconjugated protein antigen.

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