

Development of Antibody-Secreting Cells and Antigen-Specific T Cells in Cervical Lymph Nodes after Intranasal Immunization

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Intranasal (i.n.) immunization with bacterial protein antigens coupled to cholera toxin B subunit (CTB) effectively induces mucosal, especially salivary immunoglobulin A (IgA), and nonmucosal antibody responses in mice. To examine the regional distribution of antigen-specific B and T cells after i.n. immunization, antibody-secreting cells and antigen-responsive T cells in cervical lymph nodes (CLN) were compared with those found after intraoral or subcutaneous (in the neck) administration of the same antigen and with T cells found in mesenteric lymph nodes (MLN) and spleen after intragastric immunization. The i.n. immunization induced predominantly IgA antibody-secreting cells in salivary glands and IgA and IgG antibody-secreting cells in the superficial and central CLN; these responses were quantitatively enhanced if the antigen was coupled to CTB. Intraoral immunization also induced IgA and IgG antibody-secreting cells in the superficial and central CLN, but only if intact cholera toxin was included as an adjuvant. In contrast, subcutaneous (neck) immunization induced IgG antibody-secreting cells mainly in the draining facial lymph nodes. CLN cell populations resembled those of MLN, except that CLN lymphocytes had higher proportions of T cells and lower proportions of B cells and a slightly higher CD4⁺/CD8⁺ ratio among T cells than the MLN lymphocytes did. T cells that proliferated in response to antigen *in vitro* were found especially in central CLN 2 days after i.n. immunization and persisted for up to 6 months, whereas after intragastric immunization, responsive T cells were not found in the MLN for up to 14 days. After culture with antigen *in vitro*, T cells from the superficial CLN of i.n. immunized mice secreted both gamma interferon and interleukin-4. Therefore, after i.n. immunization, superficial and central CLN represent sites of regional lymphocyte development, and the central CLN in particular appear to be sites where memory T cells persist.

The majority of studies on mucosal immunization, intended to generate secretory immunoglobulin A (S-IgA) antibodies at mucosal surfaces and in secretions, have utilized the peroral or enteric route of antigen administration, which stimulates the gut-associated lymphoid tissues (GALT). However, it has become clear that there are additional routes for stimulating the common mucosal immune system, involving other inductive sites in the respiratory tract, comprising the organized lymphoid tissues in the bronchi and nasopharynx. In particular, intranasal (i.n.) immunization in both experimental animals and human subjects is a topic of considerable recent interest (13, 15, 24, 39, 40, 43, 45). Although in most cases the cellular route and mechanism of antigen uptake have not been demonstrated, the nasal lymphoid tissue (NALT) that has been described in rodents is probably important in generating these mucosal immune responses in animals (19, 20, 45). Rodent NALT has been proposed as the functional equivalent of Waldeyer's ring in humans (19), which consists of the various tonsillar and adenoid structures situated in the pharynx, and

some studies have addressed the role of Waldeyer's ring in generating human mucosal immune responses (5, 29, 30, 35). We have previously found that i.n. immunization with antigen conjugated to cholera toxin B subunit (CTB) plus cholera toxin (CT) as an adjuvant is more efficient than peroral immunization for inducing S-IgA antibodies in various secretions as well as for inducing circulating antibodies (45). CT is known to be a potent mucosal immunogen and adjuvant (4, 7, 10, 21, 22), but it is not feasible to use CT in human vaccines due to the highly toxic effects of the A1 subunit. The nontoxic CTB alone, however, seems to be ineffective as an adjuvant for intragastric (i.g.) immunization (8, 33), although it has been used via the i.n. route with apparent success when given with soluble antigens or viruses (18, 44, 45).

Numerous studies have demonstrated that GALT is a major inductive site of the common mucosal immune system for generating mucosal immunity. After encounter with antigen, sensitized T and B cells emigrate from GALT via the draining mesenteric lymph nodes (MLN) and recirculate prior to homing to effector sites within the mucosal immune system, such as the intestinal lamina propria and stroma of exocrine glands (23). It has been proposed that NALT has a similar function in disseminating antigen-sensitized lymphocytes to mucosal and also nonmucosal lymphoid tissues through the cervical lymph nodes (CLN), which drain the head and neck area (13, 41). We have found that after i.n. immunization, which is particularly effective in generating antibody responses in saliva (45), specific antibody-secreting cells may be found not only in the NALT (44) but also in the CLN to a greater extent than in the MLN, as well as in the salivary glands (45). Furthermore, after i.n. immunization, T cells isolated from NALT proliferate in

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vitro in response to antigen and express mRNA for type 1 and type 2 cytokines (44), but it has not been possible to assess cytokine secretion or activity because of limitations in the numbers of cells obtained from this tissue. However, these observations have led us to consider that the CLN could serve as a reservoir of sensitized T and B cells derived from NALT that might be responsible for sustained antibody production in the salivary glands (12, 33, 45). As different nodes within the CLN group drain different parts of the head area (41), we have examined the distribution of antibody-secreting cells after application of antigen via different routes within the head and neck region (i.n., intraoral, and subcutaneous) and have determined the lymphocyte phenotypes in these nodes. To investigate the development of T-cell help as well as memory, we have further analyzed, at various intervals after i.n. immunization, the proliferative responses and cytokines secreted by antigen-specific T cells from CLN upon stimulation *in vitro*.

MATERIALS AND METHODS

Immunization. BALB/c mice were bred and maintained in a pathogen-free colony supervised by the Animal Resources Program, University of Alabama at Birmingham, in full compliance with National Institutes of Health, guidelines for animal care. All studies were approved by the Institutional Animal Care and Use Committee.

Streptococcus mutans surface protein AgI/II (32) was conjugated to CTB at equimolar ratios by using *N*-succinimidyl-(3-[2-pyridyl]dithio)propionate as described previously (33). CT and CTB were purchased from List Biological Laboratories, Inc. (Campbell, Calif.). Mice of both sexes, 12 to 27 weeks of age, were immunized i.n. with 15 μ g of AgI/II-CTB or 10 μ g of AgI/II or i.g. with 30 μ g of the conjugate plus 5 μ g of CT; all doses were given three times at 10-day intervals (45). For intraoral immunization, 30 μ g of AgI/II-CTB with or without 5 μ g of CT was applied to the sides and bottom of the oral cavity three times at 10-day intervals. Subcutaneous immunizations of 30 μ g of AgI/II in 20% magnesium aluminum hydroxide suspension were given in the neck area twice at a 21-day interval. For enzyme-linked immunospot (ELISPOT) assay of specific antibody-secreting cells in lymph nodes and spleen, mice were killed 4 to 5 days after the last immunization. For proliferation experiments, mice were killed 2 or 14 days or 6 months after the last immunization, and superficial, central, and facial CLN (20, 41) and spleens were isolated from i.n. immunized mice, and MLN and spleens were isolated from i.g. immunized mice. For cytokine assays, mice were killed 2 days after the last immunization.

Isolation of cells. The CLN were identified as facial CLN close to the ventral posterior edge of the parotid glands, superficial CLN adjacent to the anterior edge of the submandibular salivary glands, and central (posterior) CLN deep within the musculature of the neck (41) and were excised. Likewise, MLN were located in the mesentery and were excised along with the spleens. Lymph nodes and spleens were teased apart with syringe needles to release lymphoid cells, and dispersed by syringing to obtain single-cell suspensions. The suspensions were filtered through nylon mesh to remove tissue debris and centrifuged on Ficoll-Histopaque (density, 1.083 g/ml) (Sigma) to remove dead cells. Submandibular and sublingual salivary glands were excised, trimmed of connective tissue and adjacent lymph nodes, and chopped into 0.2-mm slices. Mononuclear cells were isolated from salivary glands by two cycles of collagenase and hyaluronidase digestion as described previously (45). All cell preparations were washed and resuspended in RPMI 1640 medium with 10% fetal calf serum and counted in a hemacytometer with trypan blue to estimate viability.

ELISPOT assay. Membrane-based 96-well microtiter plates (Millititer HA; Millipore Corp., Bedford, Mass.) were coated with 5 μ g of AgI/II per ml or 10% fetal calf serum (control), diluted in borate-buffered saline (pH 8.2) overnight at room temperature. Plates were blocked with 10% fetal calf serum in RPMI 1640 at 37°C. The spleen, superficial CLN, central CLN, facial CLN, or salivary gland cell suspensions were incubated in the plates in duplicate wells at a range of concentrations appropriate for the particular assay (1×10^3 to 2×10^5 cells per well), for 3 to 4 h at 37°C in humidified 5% CO₂-air. The spots were developed with peroxidase-labeled antibodies to mouse Ig isotypes applied at previously determined optimal dilutions and 3-amino-9-ethylcarbazole-H₂O₂ substrate (33). Spots were counted under a stereo dissecting microscope in wells yielding approximately 20 to 100 spots and expressed as the number of spot-forming cells per 10^6 cells plated (33).

Proliferation assay. Cells from lymph nodes and spleens were incubated in triplicate for 3 or 4 days in 96-well U-bottom tissue culture plates (10^5 cells/well) in RPMI 1640 complete medium (supplemented with 10% fetal calf serum, 100 mM sodium pyruvate, 200 mM glutamine, nonessential amino acids, 12.5 mM HEPES, penicillin, and streptomycin) with different concentrations of concanavalin A (ConA) or AgI/II. At 16 to 20 h before being harvested, the cells were pulsed with 0.5 μ Ci of [³H]thymidine, incorporation was determined by liquid scintillation counting, and the stimulation index was calculated as the ratio

of the counts per minute (stimulated cultures) to the mean counts per minute (control cultures).

Fluorescence-activated cell sorter analysis. Cells from different organs were stained with the following pairs of fluorochrome-labeled antibodies: anti-CD3 (clone 145-2C11) and anti-B220 (clone RA-3-3A1/6.1); anti-CD3 and anti- $\alpha\beta$ TcR (clone H57-597); or anti-CD4 (clone GK1.5) and anti-CD8 (clone 53-6.72). The antibodies were supplied by the Flow Cytometry Core Facility and derived from hybridomas obtained from the American Type Culture Collection. Paired antibodies were labeled with either fluorescein or biotin; the biotinylated antibody was applied first by incubating the mixture for 20 min at 4°C in 5% fetal calf serum in Dulbecco's phosphate-buffered saline with 0.02% NaN₃. After the mixture was washed, the fluoresceinated antibody was similarly applied together with phycoerythrin-avidin. The cells were washed and finally fixed in 1% paraformaldehyde. For each sample, 5,000 gated cells identified as lymphocytes by forward- and side-scatter parameters were analyzed in a FACStar IV (Becton Dickinson) in the Flow Cytometry Core Facility.

Cytokine assays. Cells were cultured for 2 to 4 days in the presence of previously determined optimal amounts of AgI/II (3 μ g/ml) or ConA (5 μ g/ml), or in the absence of stimulus, and the supernatants were tested for cytokine activities as described below. The assays were developed and validated in the Cytokine Core Facility (director, K. W. Beagley) and routinely tested for specificity for the respective cytokines by inhibition with appropriate neutralizing antibodies.

(i) **IFN- γ bioassay.** Serial dilutions of culture supernatant were incubated with WEHI 279 cells (10^4 per well) (31) for 66 h, and 10 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; 5 mg/ml) was added for another 6-h incubation. After acidification with isopropanol-HCl, the plates were read at 415 nm and at 650 nm (25). Specificity was ensured by means of anti-gamma interferon (IFN- γ) antibody XMG1.2 (6).

(ii) **IL-4 bioassay.** CT 4S cells (5×10^3 per well) (17) were incubated with serial dilutions of test supernatant for 48 h, pulsed with 0.5 μ Ci of [³H]thymidine overnight, and counted for ³H uptake. A known concentration of interleukin-4 (IL-4) supernatant, calibrated against baculovirus-derived recombinant murine IL-4 (generously provided by W. E. Paul, National Institutes of Health, Bethesda, Md.), was assayed on the same plate as a standard for calculating the concentration of IL-4 in samples. Specificity was ensured by means of anti-IL-4 antibody 11B11 (16).

(iii) **IL-5 enzyme-linked immunosorbent assay.** Supernatants from cell cultures were serially diluted in duplicate from 1:2 dilutions and incubated overnight at 4°C in plates coated with monoclonal anti-IL-5 antibody (TRFK5; 1 μ g/ml) (36). The plates were developed with 1 μ g of biotinylated TRFK4 monoclonal antibody per ml (36) and then with avidin-peroxidase (1 μ g/ml), and the color obtained with 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)-H₂O₂ substrate was read at 414 nm. For calibration, a reference supernatant containing known concentrations of recombinant IL-5 (derived from plasmid-transfected X63Ag8.653 cells) was assayed on the same plate (1).

RESULTS

Distribution of antibody-secreting cells among CLN. To determine the location of specific antibody-secreting cells, mice were immunized either i.n. or s.c. (in the neck) with AgI/II, and three groups of CLN (superficial, central, and facial) were excised 4 days after the last dose and the cells examined by ELISPOT assay. Spleens and salivary glands were also examined for antibody-secreting cells as representative sites of systemic and mucosal antibody secretion, respectively. Other groups of mice were immunized i.n. with AgI/II-CTB conjugate, which is known to induce strong salivary IgA antibody responses (45). Mice immunized i.n. with AgI/II alone generated specific IgA antibody-secreting cells in the salivary glands and also in the superficial and central CLN but very few (10^6 cells) in the facial CLN (Fig. 1a). IgG antibody-secreting cells were also found in superficial and central CLN, and few IgG or IgA antibody-secreting cells were found in the spleen (Fig. 1a). In contrast, mice immunized s.c. with AgI/II alone showed predominantly IgG antibody-secreting cell responses in all CLN, particularly in the facial CLN (Fig. 1b), and also in the spleen. IgA antibody-secreting cells were detectable in the CLN but at a much lower level than were IgG antibody-secreting cells. Mice immunized i.n. with AgI/II conjugated to CTB generated enhanced mucosal IgA antibody responses to AgI/II as revealed by large numbers of IgA antibody-secreting cells in salivary glands (Fig. 1c), as shown previously (45). Increased numbers of cells secreting IgG or IgA antibodies to AgI/II

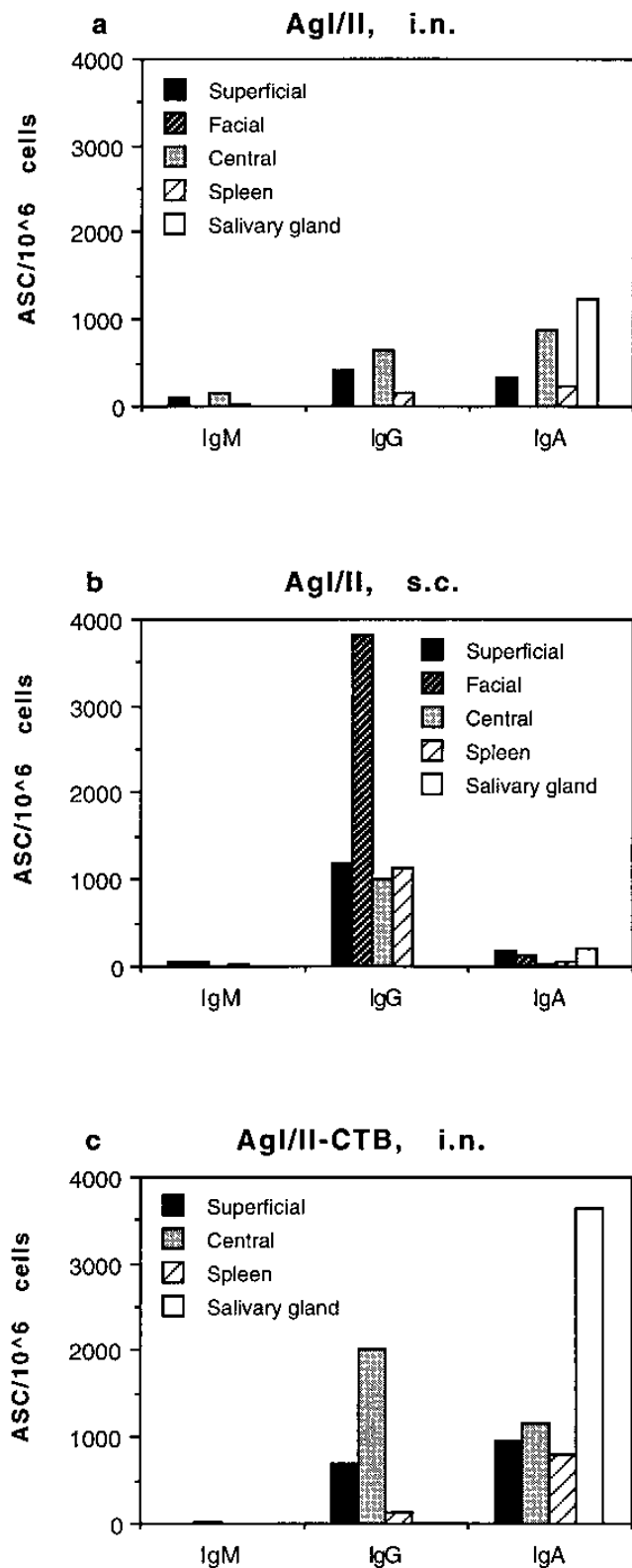


FIG. 1. AgI/II-specific antibody-secreting cells (ASC) in the superficial, facial, and central CLN, spleen, and salivary gland after immunization with AgI/II i.n. (a), AgI/II s.c. (in the neck) (b), or AgI/II-CTB conjugate i.n. (c), as determined by the isotype-specific ELISPOT assay.

were also found in both superficial and central CLN of mice immunized with AgI/II-CTB i.n. compared with AgI/II alone i.n. (Fig. 1c).

In numerous experiments on i.n. immunized mice, we observed that the superficial and central (but not facial) CLN were enlarged, suggesting that cell proliferation or accumulation was occurring. In contrast, the facial CLN were enlarged after s.c. immunization in the neck area, and, furthermore, s.c. injection (in the neck) of colloidal carbon revealed selective accumulation of carbon particles in the facial nodes. Together with the results of ELISPOT analysis, these data indicate that the facial CLN, which are known to drain the superficial area of the head and neck in rats (41), are involved in immune responses generated by s.c. injection in the head and neck region whereas the superficial and central CLN are involved in immune responses induced within the nasal passages.

Comparison of i.n. and intraoral immunization. Although i.n. immunization was performed with a small volume of immunogen (10 to 15 μ l) to limit its spread beyond the nasal passages, it is possible that some of the immunizing dose entered the oral cavity or gastrointestinal tract. To determine if such spread could account for the ensuing immune response, we immunized mice intraorally with AgI/II-CTB conjugate alone or together with CT as an adjuvant and assayed cells secreting antibody to AgI/II or CT in the CLN and spleen. IgM, IgG, and IgA antibody-secreting cells were found in the superficial and central CLN and also in the spleen (Table 1), but these responses depended upon the addition of CT as adjuvant; AgI/II-CTB alone induced only insignificant numbers of antibody-secreting cells in the CLN or spleen. Furthermore, the levels of antibody-secreting cell responses to intraoral administration of 30 μ g of AgI/II-CTB plus 5 μ g of CT were substantially (5- to 20-fold) lower than those obtained by i.n. immunization with 15 μ g of AgI/II-CTB without added CT (Fig. 1c), and even lower than the responses to 10 μ g of AgI/II alone given i.n. (Fig. 1a). Previous studies have shown that the response to i.g. immunization also depends on the addition of CT as an adjuvant and is lower than that resulting from i.n. immunization (33, 45). These data suggest that although the superficial and central CLN also drain the oral cavity, effective responses to intraoral immunization depend upon coadministration of CT adjuvant. Since i.n. immunization was not dependent upon the addition of intact CT (45), this argues against the possibility that immune responses in the CLN after i.n. immunization were due to antigen entering the oral cavity or being swallowed into the gastrointestinal tract.

Cell types in CLN and MLN. The above results indicate that the superficial and central CLN may be related to the NALT as the MLN are related to the gut-associated lymphoid tissues; i.e., both are draining lymph nodes of the respective inductive sites where responding lymphocytes may be found. Therefore, we compared the major lymphoid cell phenotypes found in these nodes, considering the different CLN separately (Table 2). CD3⁺ T cells were predominant, and CD3 was coexpressed with $\alpha\beta$ TcR in all these lymph nodes. MLN contained significantly larger numbers of B cells and fewer T cells than did facial CLN and a higher proportion of CD8⁺ T cells than did either superficial or facial CLN (Table 2), but overall these differences may not be biologically significant. The three groups of CLN showed very similar cell phenotype distributions despite having some functional differences, as described above and in the next section.

Memory responses. To detect the presence of cells sensitized to AgI/II in the draining lymph nodes at various times after i.n. or i.g. immunization, lymphoid cell suspensions were cultured with various doses of AgI/II for 4 days. Two days after

TABLE 1. Comparison of antibody-secreting cells in spleen and superficial, central, and facial CLN of mice immunized introrally with AgI/II-CTB conjugate with or without CT as adjuvant

Organ	Adjuvant	Mean \pm SD anti-AgI/II ASC/10 ⁶ cells ^a			Mean \pm SD anti-CT ASC/10 ⁶ cells ^a		
		IgM	IgG	IgA	IgM	IgG	IgA
Superficial CLN	None	2 \pm 2	0 ^b	0	1 \pm 2	0	0
	CT	63 \pm 31	135 \pm 92	55 \pm 25	115 \pm 79	237 \pm 51	117 \pm 83
Central CLN	None	1 \pm 2	0	0	3 \pm 3	0	0
	CT	83 \pm 58	217 \pm 182	230 \pm 131	97 \pm 75	367 \pm 156	277 \pm 136
Facial CLN	None	3 \pm 3	0	0	5 \pm 5	0	2 \pm 3
	CT	3 \pm 3	0	2 \pm 3	0	0	5 \pm 0
Spleen	None	5 \pm 9	0	0	0	1 \pm 2	2 \pm 2
	CT	79 \pm 18	48 \pm 11	235 \pm 66	38 \pm 33	79 \pm 39	124 \pm 112

^a Mean number of antibody-secreting cells (ASC) per 10⁶ cells ($n = 3$).

^b <1 antibody-secreting cell per 2 \times 10⁵ cells plated.

the last immunization, central (but not superficial) CLN cells from i.n. immunized mice showed stronger proliferative responses to AgI/II than did MLN cells from i.g. immunized mice (Fig. 2a and b), whereas spleen cells from both groups of mice showed weak or no responses to antigen stimulation in vitro. Similarly, 14 days after the last dose, the proliferative response to AgI/II was strong in central CLN cells of i.n. immunized mice, and MLN cells from i.g. immunized mice now also responded to AgI/II, but spleen cell responses remained weak in both groups (Fig. 2c and d). These data indicate that AgI/II-specific memory cells reside in NALT-draining central CLN of i.n. immunized mice and in GALT-draining MLN of i.g. immunized mice, at least in the short term. However, 6 months after immunization, central CLN cells of i.n. immunized mice and MLN and spleen cells of i.g. immunized mice responded similarly to AgI/II in vitro (Fig. 2e and f). Cells from the superficial CLN did not respond significantly to AgI/II at 2 or 14 days or 6 months after immunization (Fig. 2a, c, and e). These data indicate that AgI/II-specific memory cells could persist in draining lymph nodes of NALT and GALT and in the spleens of these immunized mice. Lymph node and spleen cells from unimmunized control mice stimulated with the same dose range of AgI/II in vitro revealed no proliferative responses (data not shown).

Cytokine production. Supernatants from AgI/II-stimulated superficial and central CLN and spleen cells of i.n. immunized mice contained IFN- γ , which resulted in strong inhibition of WEHI 279 cell growth (Fig. 3a to c). However, supernatants from AgI/II-stimulated MLN and spleen cells from i.g. immunized mice contained little or no IFN- γ activity (Fig. 3d and e). IFN- γ production, in both control and ConA-stimulated cul-

tures, appeared to be stronger in tissues from i.n. immunized mice than in those from i.g. immunized mice.

IL-4 was detected in AgI/II-stimulated culture supernatants of superficial (but not central) CLN cells of i.n. immunized mice, but IL-4 production in response to mitogen (ConA) stimulation of these cells was not much higher (Table 3). However, in spleen cell cultures from these mice, ConA stimulated considerably greater IL-4 production than did AgI/II (Table 3). These findings suggest that while the spleen cells may have greater total potential than CLN cells for IL-4 production under mitogen stimulation, IL-4 production by superficial CLN cells of i.n. immunized mice was largely attributable to specific antigen-sensitized cells. In contrast, in supernatants of AgI/II-stimulated MLN and spleen cell cultures from i.g. immunized mice, IL-4 was almost undetectable (Table 3), although IL-4 was produced in response to ConA. IL-5 was virtually undetectable in supernatants of AgI/II-stimulated cells of any of the tissues examined from i.n. or i.g. immunized mice, although it was induced after 2 to 3 days by ConA in superficial CLN, MLN, and spleen cell cultures (data not shown).

DISCUSSION

We found that i.n. immunization with AgI/II-CTB conjugate generated IgG and IgA antibody-secreting cells in the superficial and central CLN but not in the facial CLN whereas s.c. immunization induced a large number of IgG antibody-secreting cells in the facial CLN but fewer antibody-secreting cells in the superficial and central CLN. These results are consistent with the lymphoid drainage patterns of the head region, as

TABLE 2. Phenotypic characterization of lymphocytes in the draining lymph nodes of NALT and GALT

Cell source	Mean % of gated lymphocytes \pm SD ($n = 3$)						
	CD3 ⁺	B220 ⁺	CD3 ⁺ /B220 ⁺	$\alpha\beta$ TcR ⁺ /CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺
Superficial CLN	79.82 \pm 1.78	19.33 \pm 1.79	4.16 \pm 0.45	99.70 \pm 0.05	59.77 \pm 1.51	25.04 \pm 1.69 ^a	2.40 \pm 0.21 ^a
Central CLN ^b	83.36	14.56	5.74	99.70	62.90	23.66	2.66
Facial CLN	82.08 \pm 2.79 ^a	17.05 \pm 2.76 ^a	4.93 \pm 1.05	99.52 \pm 0.06	59.19 \pm 2.70	25.52 \pm 0.96 ^a	2.32 \pm 0.08 ^a
MLN	73.90 \pm 4.10	24.79 \pm 3.92	3.05 \pm 0.66	99.41 \pm 0.30	57.09 \pm 2.70	19.49 \pm 0.97	2.93 \pm 0.03

^a Significantly different from corresponding MLN value ($P < 0.05$, unpaired t test).

^b Pooled from five mice.

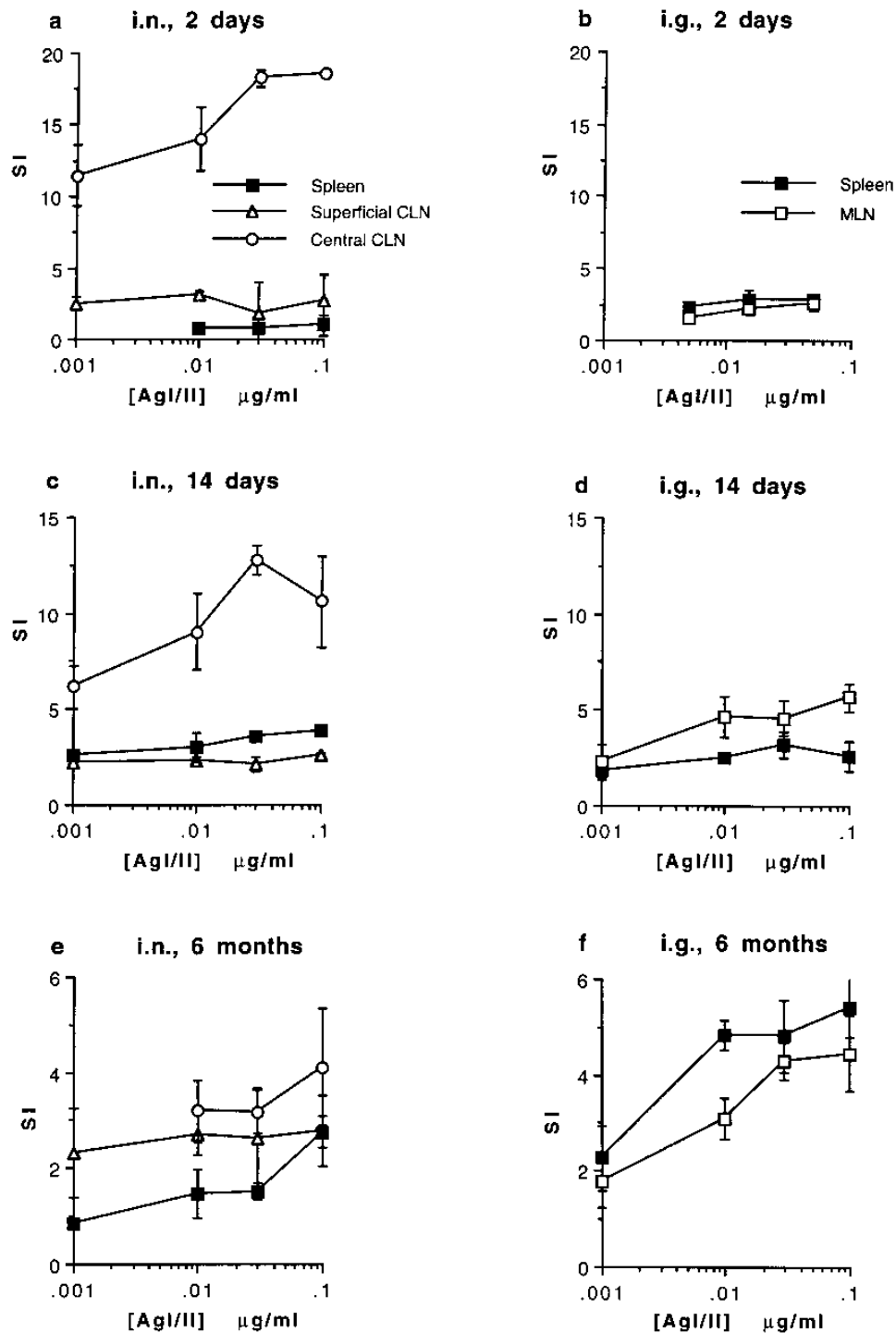


FIG. 2. Proliferative responses of cells from superficial and central CLN, MLN, and spleens cultured in vitro with AgI/II for 4 days. Mice were immunized with AgI/II-CTB i.n. (a, c, and e) or with AgI/II-CTB plus CT adjuvant i.g. (b, d, and f), and organs were taken 2 days (a and b), 14 days (c and d), or 6 months (e and f) after the third immunization dose. Results are shown as stimulation index (SI) determined by ^3H thymidine incorporation. Background ^3H thymidine incorporation in unstimulated cultures was as follows: (a) spleen, $9,352 \pm 880$ cpm; superficial CLN, $7,257 \pm 4,745$ cpm; central CLN, $1,747 \pm 36$ cpm; (b) spleen, $6,464 \pm 3,346$ cpm; MLN, $4,965 \pm 667$ cpm; (c) spleen, $3,627 \pm 519$ cpm; superficial CLN, $11,884 \pm 1,735$; central CLN, $2,346 \pm 1,312$ cpm; (d) spleen, $4,496 \pm 965$; MLN, $2,352 \pm 282$; (e) spleen, 856 ± 232 cpm; superficial CLN, $1,546 \pm 698$ cpm; central CLN, $1,097 \pm 668$ cpm; (f) spleen, $1,085 \pm 651$ cpm; MLN, $1,320 \pm 174$ cpm.

illustrated by the injection of colloidal carbon s.c. in the neck area, which resulted in staining of the facial CLN but not of the superficial or central CLN. NALT, a bilateral strip of nonencapsulated lymphoid tissue at the base of the nasal passages,

has been described as the functional equivalent in rats and mice of Waldeyer's ring in humans (20, 42). As uptake of particles may occur through M-like cells that overlie NALT (38, 42), this most probably represents the site of stimulation

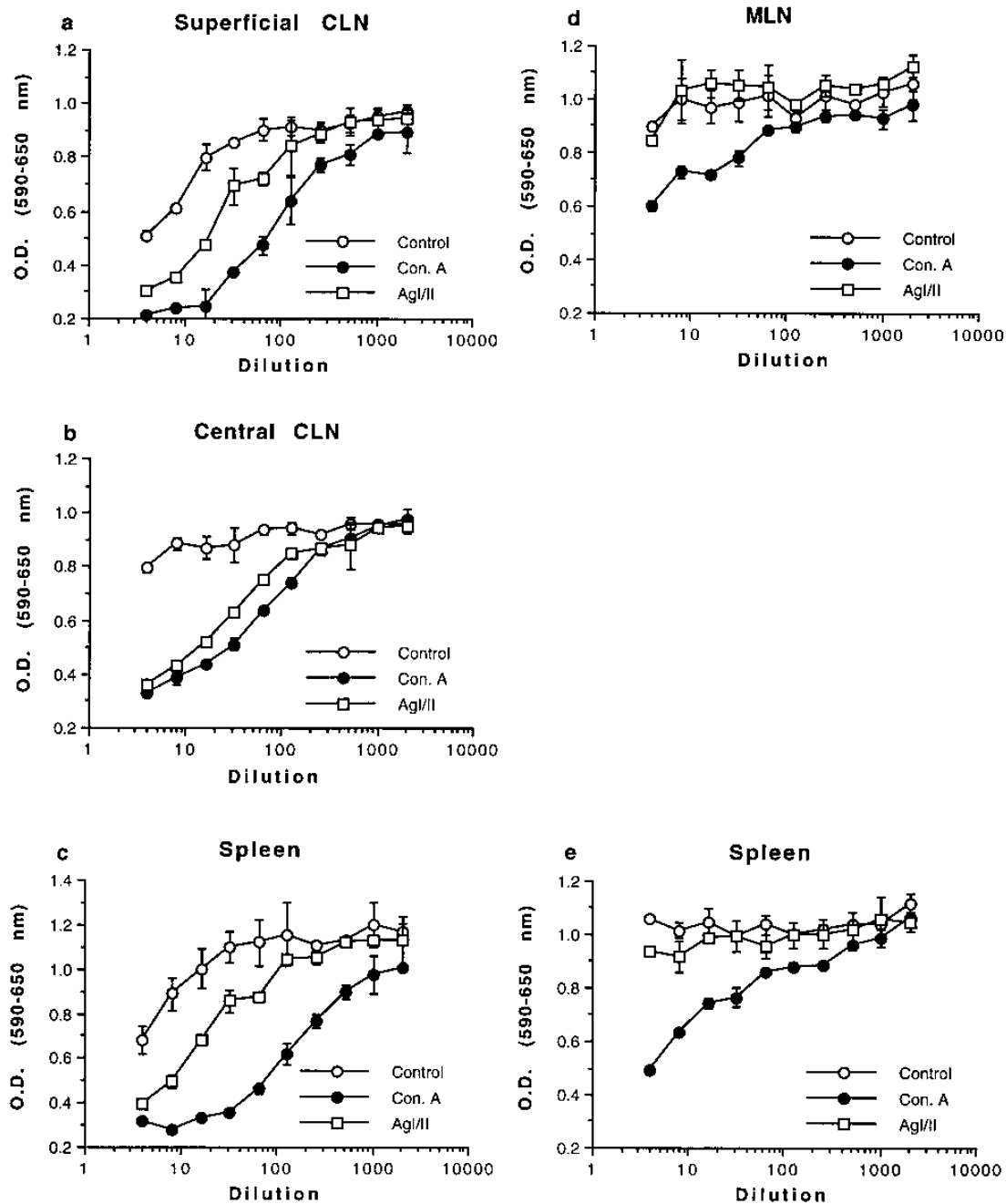


FIG. 3. IFN- γ assay of supernatants of cells from central and superficial CLN, MLN, and spleens cultured in vitro with AgI/II for 4 days. Mice were immunized with AgI/II-CTB i.n. (a to c) or with AgI/II-CTB plus CT adjuvant i.g. (d and e), and organs were taken 2 days after the third immunization dose. Cells were cultured in vitro with AgI/II (3 μ g/ml) or ConA (5 μ g/ml) or without stimulation, and dilutions of supernatants were bioassayed by inhibition of WEHI 279 cell growth, determined by MTT reduction. O.D., optical density.

after i.n. immunization, but uptake of soluble antigen may also occur through the general nasal epithelium (20), especially if its permeability is increased by CT or CTB (11). As studies with rats have shown that soluble proteins injected into NALT are recoverable in the central CLN (2, 14), it has been proposed that lymphoid drainage from NALT runs directly to the central CLN whereas the nasal submucosa appears to drain first to the superficial CLN, which in turn drain to the central CLN (20, 41). If this pattern of cell traffic also occurs in mice,

the increased permeability of the nasal mucosa induced especially by CT (11) may lead to increased responses in the superficial CLN and may thereby account for the promotion of IgE responses by CT (37, 46) due to the influence of IL-4, which was produced by cells from the superficial but not the central CLN. However, the production of IFN- γ , which suppresses Th2 cells and the production of IL-4, in both sets of CLN could serve to counteract such tendencies.

We previously reported that i.n. immunization with AgI/II

TABLE 3. IL-4 concentrations in culture supernatants of cells from CLN and spleen cells of i.n. immunized mice and from MLN and spleen cells of i.g. immunized mice

Immunization	Cell source	Stimulation	IL-4 concn (U/ml) after culture for:		
			18 h	72 h	96 h
i.n.	Superficial CLN	None	0	0	0.95
		AgI/II ^a	0.39	0.43	0.96
		ConA ^b	0.94	1.02	1.65
	Central CLN	None	ND ^c	0	ND
		AgI/II	ND	0	ND
		ConA	ND	1.13	ND
	Spleen	None	0	0.35	0.27
		AgI/II	0.55	0.97	0.61
		ConA	12.3	20.6	21.1
i.g.	MLN	None	0	0	0
		AgI/II	0	0	0
		ConA	0	2.69	2.86
	Spleen	None	0	0	0
		AgI/II	0.23	0	0
		ConA	0.71	9.03	9.5

^a 3 µg/ml.

^b 5 µg/ml.

^c ND, not determined.

alone induces weak but measurable salivary IgA and serum IgG antibody responses and that both serum and secretory responses are substantially enhanced when AgI/II is either conjugated to or mixed with CTB (45). These findings are reflected in the present experiments in the induction of IgG and IgA antibody-secreting cells superficial and central CLN and of IgA antibody-secreting cells in salivary glands by i.n. immunization with AgI/II. These responses were elevated, but the relative distribution of antibody-secreting cells was not altered, when AgI/II was conjugated to CTB. In contrast, i.n. immunization of rats is reported to induce only modest serum antibody responses unless the animals are first primed s.c. (14) and to generate antibody-secreting cells only in the posterior (central) CLN and spleen but not in NALT (13).

Intraoral immunization with AgI/II-CTB conjugate plus CT induced strong responses in the superficial and central CLN, indicating that these nodes also drain the oral cavity, although the exact site of immunogen uptake and induction of the immune response is not clear. However, the requirement for adding CT adjuvant to generate responses by the intraoral route but not by the i.n. route implies that different sites of antigen uptake and stimulation are involved, even if the mechanism is unknown. Previous studies have shown that intra-oral immunization with AgI/II-CTB plus CT is effective in inducing salivary IgA as well as serum IgG antibody responses (34). Although we cannot eliminate the possibility that some immunogen was swallowed and thereby reached the GALT, the observation that the CLN were swollen and contained specific antibody-secreting cells and T cells strongly indicates that the induction of the responses took place in lymphoid tissues within the head and neck region, because these lymph nodes do not respond to enteric immunization (45). Besides the lingual tonsils found in primates (27), lymphoid tissue associated with the ducts of minor salivary glands in the mouth has been proposed to be a site of antigen uptake and immune response

induction (28), and similar tissue is also present in mice (3). Although the exact site of antigen uptake is uncertain, i.n., intraoral, and i.g. immunization all induce responses in corresponding mucosal draining lymph nodes, resulting in the development of specific IgA antibody-secreting cells in the salivary glands and other mucosal tissues and of IgA antibodies in the corresponding secretions (45). Thus, the CLN have the same relationship to NALT and oral lymphoid tissue as do the MLN to GALT, and both sets of lymph nodes appear to be intermediate points of lymphocyte differentiation in the common mucosal immune system that disseminates responses to mucosal effector sites.

In vitro restimulation of central CLN and MLN cells obtained after i.n. or i.g. immunization, respectively, with AgI/II either immediately (2 days) or in the short term (2 weeks) or long term (6 months) after immunization showed that central CLN cells displayed earlier and stronger proliferative responses than did MLN. The stronger responses of CLN cells may be related to the finding that i.n. immunization generally results in higher antibody responses in serum and secretions than does i.g. immunization, even though smaller doses of AgI/II-CTB are given i.n. and without the addition of CT as a mucosal adjuvant (45). Although numerous factors undoubtedly contribute to these differences, including the exposure of i.g. administered immunogens to gastric acid and intestinal enzymes, such factors do not readily account for the difference in timing of proliferative responses in CLN and MLN cells: CLN cells responded much earlier (within 2 days) than MLN cells, yet by 6 months the proliferative responses in CLN, MLN, and spleen in the two immunization groups had become similar. The corresponding inductive sites, NALT and GALT, display differences in the cell populations: in particular, NALT has a higher T-cell/B-cell ratio, a lower CD4⁺-cell/CD8⁺-cell ratio, and an overall more naive state of lymphocyte development than do Peyer's patches (44), probably reflecting a substantially higher antigenic load with consequently greater competition between antigens in GALT. After stimulation by antigen, NALT seeds T and B cells to the draining CLN, where large numbers of specific antibody-secreting cells were found, along with antigen-specific T cells especially in the superficial CLN and antigen-specific proliferating cells mainly in the central CLN.

Cells from both superficial and central CLN secreted more IFN-γ than did MLN cells after stimulation with AgI/II, indicating higher frequencies of antigen-sensitized cells capable of secreting IFN-γ in the draining lymph nodes of NALT than in those of GALT. In addition, superficial CLN cells secreted IL-4 in response to AgI/II stimulation whereas, in contrast, central CLN cells proliferated but did not secrete detectable IL-4. Mixed patterns of cytokine mRNA expression were also found in NALT cells of i.n. immunized mice (44) and may be indicative of Th0 or mixed type 1 and 2 patterns of T-cell help (9, 26), but it is now accepted that more diverse states of T helper cells exist. These findings may be related to the similar proportions of IgG and IgA antibody-secreting cells found in the CLN, in comparison with NALT, where IgA antibody-secreting cells predominate (44), since IFN-γ is known to enhance IgG2 responses in mice whereas IL-4 promotes IgG1 responses. It is possible that NALT-derived antigen-stimulated B cells become switched mainly to IgG production in the CLN whereas IgA isotype switching appears to occur in the NALT. The pattern of cytokine production in the draining lymph nodes of NALT would then reflect the circulatory rather than the mucosal aspect of i.n. induced responses. In support of this, we have found large numbers of IgA antibody-secreting cells in the NALT of i.n. immunized mice (44), and we postulate that

these migrate rapidly through CLN en route to the effector sites of mucosal immunity such as the salivary glands and gut lamina propria, where they may be recovered (45). We previously noted that the numbers of IgA antibody-secreting cells in MLN decline rapidly from 3 days after i.g. immunization (33). If this applies also for IgA antibody-secreting cells in CLN, the persistence of IgG antibody-secreting cells and their corresponding T helper cells in these lymph nodes, which do not belong exclusively to the mucosal immune system, might account for our observations.

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