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Induction of Compartmentalized B-Cell Responses in Human Tonsils

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The capacity of tonsillar and nasal mucosal lymphoid tissues to serve as induction sites of local and/or distant B-cell responses in humans has been examined. The frequencies of vaccine-specific antibody-secreting cells (ASC) in cell suspensions from palatine tonsils (PT) and adenoids were determined after local (intratonsillar [i.t.]) and regional (intranasal [i.n.]) immunizations as well as peroral and parenteral immunizations with cholera and tetanus toxoids. While peroral and parenteral immunizations evoked negligible ASC responses in PT, i.t. vaccination induced a substantial ASC response which consisted of immunoglobulin G (IgG) and IgA ASC. Responses were highly restricted to immunized tonsils. Primary immunization in one PT followed by a second immunization of both PT evoked a larger ASC response in the primed tonsil. The latter ASC response was associated with higher frequencies of ASC presursors in primed tonsils. Furthermore, two i.n. immunizations induced only modest ASC responses in PT, although such immunizations evoked high ASC responses in adenoids. However, both i.t. and i.n. routes of immunization induced specific peripheral blood ASC responses, suggesting that a fraction of B cells activated in tonsils or in nasal mucosa may enter the circulation and disseminate to distant organs. These blood ASC responses preceded increases in both IgA and IgG antibody titers in nasal washes and serum samples. However, vaccine-specific ASC were not detected in duodenal cell suspensions from volunteers who had received i.t. or i.n. immunizations. Collectively, these results indicate that tonsils can serve as expression sites of locally induced antibody responses and support the development of immunological memory. Furthermore, tonsils may serve as powerful inductive sites for immune responses expressed in the upper aerodigestive tract.

The palatine tonsils (PT) and adenoids, also known as nasopharyngeal tonsils, are organized lymphoepithelial structures that contain all the cell types necessary to support humoral and cell-mediated immune responses (1, 2). Equipped with a specialized epithelium consisting of antigen-transporting M cells (10) and with lymphoid follicules containing germinal centers (2), tonsils have been shown to be sites of intense B-cell maturation and differentiation associated with local T-cell activation (2, 3, 5, 7, 19). The early observation that tonsillectomy reduces preexisting antibody titers in nasopharyngeal fluids (17) together with recent findings that demonstrate preferential engraftment of human tonsillar B cells into the lungs of mice with severe combined immunodeficiency (15, 16) has led to the notion that tonsils may represent a functional analog of intestinal Peyer's patches for the upper aerodigestive tract (1, 11).

These considerations prompted us to examine antigen-specific B-cell responses in human tonsils after immunizations at local (intratonsillar [i.t.]), regional (intranasal [i.n.]), and remote mucosal (peroral [p.o.]) sites as well as after systemic immunization.

MATERIALS AND METHODS

Volunteers and immunizations. With due approval from the Ethical Committee on Human Experimentation of the Medical Faculty at the University of Göteborg, 27 Swedish individuals (16 males and 11 females, aged 16 to 37 years) entered the study under informed consent. Each patient had been scheduled for tonsillectomy on the basis of recurrent tonsillitis or severe sleep-induced apnea. Separate groups of volunteers were immunized according to the following routes and regimens of vaccination.

(i) p.o. immunization. Seven volunteers (all of them suffered from recurrent tonsillitis) received two doses of an oral cholera vaccine (9) given 2 weeks apart; each dose consisted of 10^{11} killed *Vibrio cholerae* organisms and 1 mg of purified cholera toxin B subunit (CTB) (Statens Bacteriology Laboratory, Stockholm, Sweden) in bicarbonate buffer.

(ii) Systemic immunization. On the day of the second oral vaccination, five orally immunized volunteers also received a subcutaneous (s.c.) injection of a tetanus toxoid (TT)-diphtheria toxoid vaccine (SBL) that contained 2 flocculating units (Lf) of TT.

(iii) i.t. immunization. Six volunteers (two with recurrent tonsillitis and four with apnea) were each given a single i.t. injection that consisted of 50 μg of purified CTB (Institut Merieux, Lyon, France) together with 0.75 Lf of TT in the form of tetanus-diphtheria vaccine injected in a total volume of 0.2 ml under the capsule of the right PT 1 week before tonsillectomy. Two other volunteers (one with recurrent tonsillitis and one with apnea) were given identical i.t. injections 2 weeks before tonsillectomy, and two volunteers (one with recurrent tonsillitis and one with apnea) were given identical i.t. injections 3 weeks before tonsillectomy. In addition, each of three volunteers (two with recurrent tonsillitis and one with apnea) received a primary i.t. immunization with CTB alone in the right tonsil and, 3 weeks later, a second immunization consisting of both CTB and tetanus-diphtheria vaccines in both tonsils.

(iv) i.n. immunization. Seven volunteers (six with recurrent tonsillitis and one with apnea) were immunized twice i.n. with 1 mg of purified CTB given 2 to 4 weeks apart. After a nasal wash with pyrogen-free saline, 1 mg of CTB was introduced into the nostrils, kept for 1 min, and then sneezed out. Care was taken to inform volunteers to avoid swallowing the vaccine. As a further precaution, the CTB immunogen was administered in saline without antacid buffering so as to prevent any residual CTB from reaching the intestine in an active (pentameric) form.

Each tonsillectomy was performed 1 week after the last immunization, unless otherwise stated, at the Ear, Nose, and Throat Department, Sahlgrenska Hospital, Göteborg, Sweden. Whole PT, adenoid biopsies, and heparinized venous blood samples were collected at surgery, and the tonsillar material was kept at 4°C in phosphate-buffered saline (PBS) until processed (within 2 h). Serum and whole saliva samples and nasal washes from four i.t. immunized and five i.n. immunized volunteers were collected before each immunization and 1 to 6 weeks

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after each immunization. Fluids were inactivated (56°C, 30 min), clarified by centrifugation, and kept frozen prior to being assayed.

From four of these volunteers who agreed to undergo gastroscopy, four to six duodenal pinch biopsies were collected at surgery after i.t. (two volunteers) and i.n. (two volunteers) immunizations.

Isolation of MNC. Parts of tonsils macroscopically judged as inflamed were removed, and remaining tissue was cut into fragments (1 by 1 mm) with a semiautomated tissue chopper (McIlwain, Gilford, United Kingdom). Fragments were then disrupted by being pressed through a 150-μm-pore-size nylon mesh, and the resulting single cell suspensions were washed three times by centrifugation in cold Ca^{2+}- and Mg^{2+}-free isotonic PBS, pH 7.4 (Gibco Europe, Edinburgh, United Kingdom). Tonsillar and peripheral blood mononuclear cells (MNC) were isolated by gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Interface MNC were collected, washed three times with cold Ca^{2+}- and Mg^{2+}-free PBS, and then resuspended in Iscove's medium (Gibco) supplemented with 5% fetal calf serum and 100 μg of gentamicin (Gibco) per ml (complete medium). Duodenal biopsies were cut into fragments (0.1 by 0.1 mm), and MNC were isolated from fragments by the two-step enzymatic dispersion procedure previously described (20). Cell suspensions were kept on ice prior to being assayed.

Limiting dilution analysis of specific antibody-secreting cell (ASC) precursors. Immunoglobulin D-negative (IgD^-) cells were isolated from tonsillar B-cell suspensions with magnetic beads (M450; Dynal, Olso, Norway) coated with mouse monoclonal antibody that reacts with human IgD (Dakopatts AS, Glostrup, Denmark). IgD^- B cells were isolated and cultured essentially as described by Hirohata et al. (8) at cell densities between 2.5×10^3 and 2×10^5 cells per well together with 10^5 irradiated (2,500 rad) anti-CD3-stimulated $CD4^+$ T cells in 96-well flat-bottom tissue culture plates (Nunc, Roskilde, Denmark). Supernatants from individual wells were collected after 8 days of culture and screened for CTB-specific antibody activity.

Detection of CTB-specific antibodies. Serum, saliva, and nasal wash samples as well as cell culture supernatants from limiting dilution experiments were screened for CTB-specific antibody activity by a solid-phase GM1 enzyme-linked immunosorbent assay (22) with the same developing antibodies as the ELISPOT assay described below. The titer was determined as the dilution that gave an absorbance of 0.4 above the background value, and a >1.5-fold difference in titer between samples was considered significant (22).

Enumeration of ASCs. MNC suspensions were assayed for the total number of cells spontaneously secreting IgG, IgA, and IgM by a two-color micromodification of the ELISPOT technique (4). Briefly, various numbers of MNC were incubated for 3 to 4 h at 37°C in 100 μl of complete medium in nitrocellulose-bottomed 96-well plates (Millipore, Bedford, Mass.) coated with goat antibodies to human immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, Pa.). Zones of solid-phase-bound antibodies secreted by individual MNC were revealed as spots by stepwise addition of combinations of horseradish peroxidase- or alkaline phosphatase-labeled goat antibodies to human IgG, IgA, and IgM (Southern Biotechnology Associates, Birmingham, Ala.) and suitable chromogen substrates that yielded insoluble products. Vaccine-specific IgG and IgA ASC were detected in a similar way in wells previously coated with 100 μl of PBS that contained 20 μg of purified TT (SBL) per ml or 2.5 μg of cholera toxin (List Biological Laboratories, Inc., Campbell, Calif.) per ml. For the latter antigen, wells were first coated with 3 μM of GM_1 ganglioside (Sigma Chemical Co., St. Louis, Mo.) to facilitate absorption of cholera toxin. For control purposes, wells exposed only to PBS that contained 1% fetal calf serum were included in all assays. The number of spots obtained in control wells was subtracted from the number of spots determined in antigen-coated wells.

Statistical methods. Differences in the ASC responses of right and left tonsils were evaluated by the Wilcoxon signed rank test, and differences between tonsil and blood immunoglobulin-secreting cell (ISC) frequencies were evaluated by the Wilcoxon rank sum test.

RESULTS

Baseline frequencies of tonsillar ISCs. The frequencies of cells secreting IgG, IgA, or IgM, regardless of antigen specificity, in MNC suspensions isolated from PT and adenoids and from peripheral blood were determined by a reverse ELISPOT assay. PT and adenoid cell suspensions contained significantly higher numbers of ISCs in all isotypes than did corresponding peripheral blood MNC suspensions ($P < 0.001$). Furthermore, whereas IgG and IgA ISCs were found in comparable proportions in blood, IgG ISCs were the predominant ISC population detected in PT and adenoids (Table 1).

Induction of local antibody responses and immunological memory in tonsils. To determine the routes of antigen delivery by which a tonsillar B-cell response could be induced, the frequencies of vaccine-specific ASCs in PT cell suspensions after two p.o., s.c., or i.n. immunizations or after a single i.t. immunization were determined. The distant mucosal (p.o.) and parenteral (s.c.) routes of immunization induced only modest specific ASC responses in PT (Fig. 1A). i.n., i.e., regional, immunization was at best poorly effective at inducing a specific ASC response in PT (Fig. 1A). However, such i.n. immunizations evoked considerably higher ASC responses in adenoids collected from the same volunteers (Fig. 1B). On average, these ASC responses consisted of comparable proportions of IgG- and IgA-secreting cells.

TABLE 1. Spontaneous ISCs in human PT and nasopharyngeal tonsils

Sample (*n*)	Mean ISCs/10^7 MNC ± SD		
	IgG	IgA	IgM
PT (26)	130,000 ± 120,000	45,000 ± 58,000	6,700 ± 5,000
Nasopharyngeal tonsils (7)	180,000 ± 210,000	60,000 ± 40,000	10,000 ± 4,000
Peripheral blood (24)	11,000 ± 12,000	5,800 ± 4,900	340 ± 1,200

In contrast to the modest PT ASC responses induced by the routes of immunization discussed immediately above, a single i.t. immunization with CTB and TT given 1 week before tonsillectomy induced ASC responses against both antigens (Fig. 1A). PT ASC responses were dominated by IgG-secreting cells, although appreciable numbers of IgA-secreting cells were also detected. MNC suspensions from PT collected 2 and 3 weeks after local immunization displayed weaker ASC responses (data not shown). ASC responses in adenoids following i.t. immunization could be detected in only two volunteers, both with low frequencies of vaccine-specific ASCs.

On the basis of the apparent dependence of tonsillar B-cell responses on local antigen exposure, the frequencies of vaccine-specific ASCs in the right and left PT were compared after

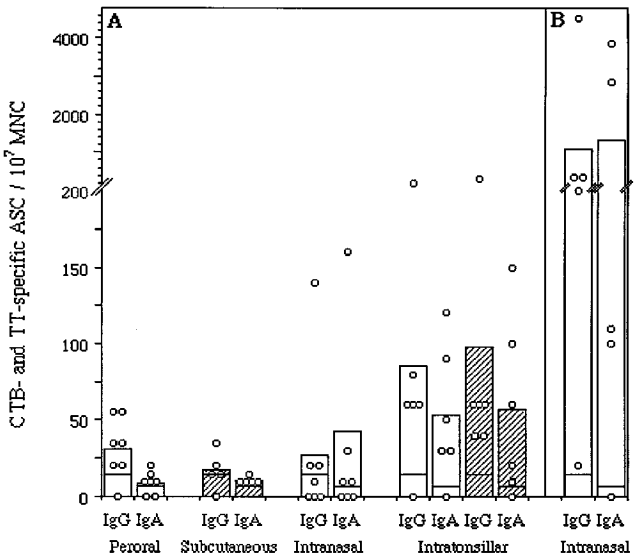

FIG. 1. Tonsillar vaccine-specific ASC responses after local and extratonsillar immunizations. ASC responses were determined 1 week after immunization. Bars indicate the geometric mean frequencies of CTB-specific (open bars) and TT-specific (hatched bars) ASCs in PT after i.t., i.n., p.o., and s.c. immunization with CTB and/or TT (A) and in nasopharyngeal tonsils after i.n. immunization with CTB (B). Circles indicate individual values, and horizontal lines represent the means + 2 standard deviations of CTB and/or TT background ASC frequencies determined for seven nonimmunized tonsil donors.

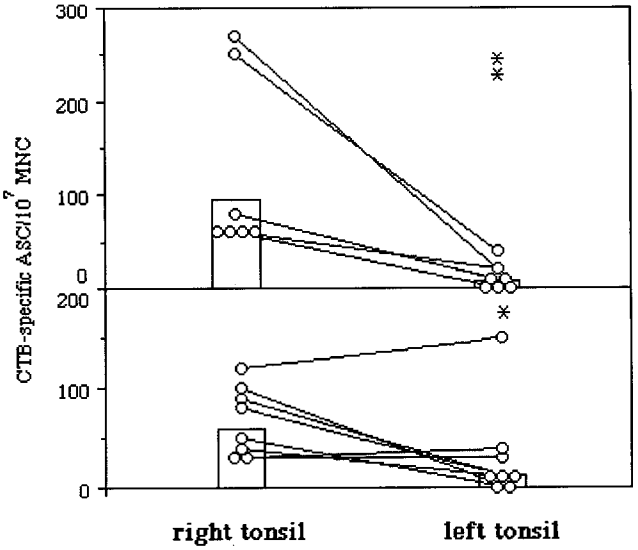

FIG. 2. Tonsillar ASC responses after local immunization with CTB. ASC responses were determined in suspensions obtained from right and left PT after the injection of CTB into the right tonsil. Connected circles indicate individual data collected from volunteers that responded to i.t. immunization with IgG ASCs (upper panel) and/or IgA ASCs (lower panel), bars indicate geometric means of responding individuals, and asterisks denote significance levels (*, $P < 0.05$; **, $P < 0.02$; Wilcoxon signed rank test).

a local i.t. injection of the right tonsil with CTB. As shown in Fig. 2, the frequencies of specific IgG- and IgA-secreting cells in the immunized (right) tonsil were significantly higher than those in the nonexposed (left) tonsil ($P < 0.02$ and $P < 0.05$, respectively).

Primary immunization of the right PT followed 3 weeks later by a second immunization of both right and left PT with CTB resulted in higher ASC responses to CTB in the right PT than in the left PT for two of three volunteers examined (Table 2). Further, the injection of both right and left PT with TT evoked comparable TT-specific ASC responses in both tonsils. To evaluate whether increased ASC responses in twice immunized tonsils were associated with local regulatory changes that allowed the expansion of specific B-cell precursors upon stimulation, a limiting dilution culture system was employed. CTB-specific ASC precursor cell frequencies in cell suspensions from the right and left tonsils of a volunteer who had been immunized in the right tonsil and subsequently in both tonsils were determined. The right tonsil contained a higher frequency (1/67,000) of IgD^- B cells that could be induced to produce CTB-specific IgG antibodies than did the left tonsil

TABLE 2. Frequencies of tonsillar CTB- and TT-specific ASCs after one immunization with CTB in the left tonsil compared with two immunizations with CTB in the right tonsil and primary immunization with TT in both tonsils

Donor	Specific ASCs/10^7 tonsillar MNC (right/left ratio)							
	CTB				TT			
	Left tonsil		Right tonsil		Left tonsil		Right tonsil	
	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
1	19	30	144 (7.6)	65 (2.2)	15	14	16 (1.1)	0 (0)
2	59	82	101 (1.7)	245 (3.0)	70	200	44 (0.6)	68 (0.3)
3	28	40	45 (1.6)	58 (1.4)	11	28	15 (1.4)	48 (1.7)

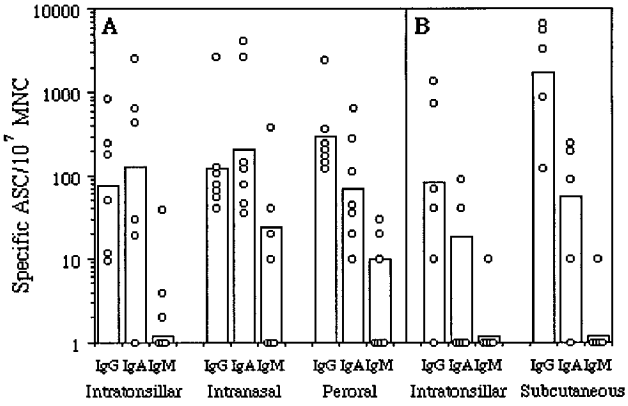

FIG. 3. Specific ASC responses in peripheral blood MNC after i.t. and i.n. immunization. Bars indicate geometric means, and circles indicate individual frequencies of circulating specific ASCs per 10^7 MNC determined 1 week after immunization with CTB (A) and TT (B). For comparison, blood ASC responses to p.o. CTB (A) and parenteral TT (B) are also depicted.

(<1/200,000). IgD^- CTB-specific IgA ASC precursors were less frequent (<1/200,000 in both tonsils).

Extratonsillar B-cell responses after i.t. and i.n. immunizations. We also examined to what extent B-cell responses induced in PT and adenoids after local (i.t.) and regional (i.n.) immunizations disseminated to distant lymphoid compartments such as the intestine and blood. Both i.t. and i.n. immunizations with CTB induced ASC responses in blood, and these responses were comparable to those seen after p.o. immunization with respect to magnitude and isotype distribution (Fig. 3A). Thus, in all but one of the volunteers examined, circulating ASC responses to CTB which consisted of comparable numbers of IgA and IgG ASCs developed. Similarly, i.t. immunization with TT induced blood ASC responses that were dominated by IgG ASCs in all of the volunteers examined, with IgA ASC less frequently observed (Fig. 3B). Although similar with respect to isotype composition, blood TT-specific ASC responses induced after s.c. immunization were considerably higher than those evoked by i.t. immunization (Fig. 3B).

Furthermore, i.t. immunization resulted in substantially increased IgG and IgA CTB-specific titers in serum samples from all volunteers at all time points analyzed (Table 3). Moreover, these immunizations induced significantly increased an-

TABLE 3. CTB-specific antibody responses in serum, saliva, and nasal wash samples after i.t. and i.n. immunizations

Immunization and sample	Frequency of responders[a]		Titer increase[b]	
	IgG	IgA	IgG	IgA
i.t.				
Serum	4/4	3/3	22–182	18–233
Saliva	3/4	1/4	2.4–6.9	28
Nasal wash	3/3	2/3	3.8–6.9	3.4–4.0
i.n.				
Serum	5/5	5/5	3.4–68	2.9–210
Saliva	1/4	1/4	5.0	11
Nasal wash	1/5	3/5	1.7	1.9–2.7

[a] An individual was considered to be a responder when IgG or IgA antibody titers induced by immunization exceeded preimmune values by at least 1.5-fold during a 6-week follow-up.

[b] Range of titer increases in relation to preimmunization titers in responding volunteers.

titoxin titers for IgG or IgA in saliva from three of four volunteers examined and in nasal washes from all of the volunteers examined. The latter responses were generally smaller and appeared later after immunization than serum responses and did not involve IgA in some volunteers (Table 3). Significantly increased IgG and/or IgA titers in serum samples from all of the i.n. immunized individuals were also found. Salivary antitoxin titers were increased for only one i.n. immunized volunteer, whereas three of five volunteers had significantly increased IgA titers in nasal washes.

Duodenal MNC suspensions were assayed for the presence of vaccine-specific ASCs after i.t. ($n = 2$) or i.n. immunization ($n = 2$) with CTB. In all four volunteers examined, neither route of immunization appeared to evoke an ASC response in the small intestine (data not shown). In all of these volunteers, an ASC response was detected in blood.

DISCUSSION

This study demonstrates that human tonsils can serve as efficient expression sites for locally induced antibody responses and are endowed with powerful inductive functions for immune responses expressed at remote sites. These features make it possible for tonsils to respond to locally encountered antigens and to supply other tissues with activated memory and effector B cells.

The fact that parenteral immunization with TT failed to induce an antibody response in tonsils demonstrates that the tonsillar immune system functions independently from the systemic immune apparatus. This conclusion is in keeping with earlier reports that showed that i.n. immunization and local tonsillar or nasal infection prime tonsillar lymphocytes more efficiently than does parenteral vaccination (14). Similarly, p.o. immunization was poorly effective at inducing an antibody response in tonsils, in contrast to local (i.t.) and regional (i.n.) immunization. This suggests that tonsils function largely independently from the lower enteromucosal immune system. The fact that tonsillar ISCs are induced to produce mainly IgG (2, 3), whereas intestinally activated ASCs preferentially secrete IgA (13), may also reflect the reciprocal independence of these lymphoid compartments. In further support of this notion, the i.t. and i.n. routes of immunization failed to evoke an antibody response in the small intestine. Moreover, the finding that i.n. immunization evoked an ASC response in the adenoids but not in PT indicates that a high degree of functional subcompartmentalization exists within Waldeyer's ring in regard to the induction of humoral immune responses. Such a notion is particularly well illustrated by the findings that i.t. immunization induced an ASC response in the injected tonsil but not, or at best inconsistently, in the contralateral one. The relatively large ASC response in adenoids after i.n. immunization compared with the ASC response in PT after i.t. immunization may reflect the fact that i.n. immunization better imitates a natural route of antigen encounter and uptake than i.t. injection of antigen.

Subcompartmentalization of humoral immune responses also appears to involve the induction of immunological memory as indicated by the findings that a second i.t. immunization led to increased ASC responses associated with enlarged ASC precursor frequencies in a previously primed tonsil compared with those of a not previously injected tonsil. Previous studies have shown that tonsillar MNC can be induced to proliferate and differentiate into ASCs upon in vitro exposure to antigens normally encountered in the upper respiratory tract but not elsewhere (6, 14, 18, 21). The results of this study confirm that tonsils can support the development of immunological memory and further indicate that such development is highly dependent on local antigen exposure.

The large number of vaccine-specific spontaneous ASCs detected in blood after i.t. immunization also indicates that tonsils may constitute an important reservoir of activated B cells capable of migrating to other tissues via efferent lymph and blood. These blood ASCs most likely represent the direct progeny of a fraction of tonsillar B-cell immunoblasts whose destination might be somewhere other than the tonsils. On the basis of the observation that i.t. injection with either CTB or TT induced a response in the injected tonsil but not in the nonexposed contralateral tonsil, it seems rather unlikely that such circulating ASCs have to transit through the entire vasculature before returning to the immunized tonsil. The observation that i.t. immunization evoked IgA and/or IgG antibody responses in salivary and nasal secretions in almost all of the volunteers examined suggests that tonsil-derived ASC precursors can seed the buccal and airway mucosae. This observation is in keeping with the seminal report of Ogra (17), who demonstrated that tonsillectomy and adenoidectomy reduced specific IgA antibody titers in nasopharyngeal secretions from children previously immunized with oral live-poliovirus vaccine. The possibility that such nasal and salivary antibody responses may reflect passive transport of antibodies formed in tonsils and/or serum appears to be unlikely since (i) intravascular polymeric IgA is not efficiently transported into human saliva, whereas a small fraction of IgG can transude from plasma to secretions (12), (ii) systemic immunization with TT, although evoking strong serum IgG antibody responses, does not induce detectable responses in external secretions (23), and (iii) increased IgA and IgG antibody levels in saliva and nasal washes do not correlate with increases in IgA and IgG antibody titers in serum samples. One potentially important destination for such circulating tonsillar ASCs might be the airway mucosa, as suggested by recent studies by Ogra's group that involved the engraftment of human tonsillar B cells in the lungs of mice with severe combined immunodeficiency (15, 16). Consistent with these studies, we could not document any detectable ASC responses in intestinal biopsies after i.t. immunization or i.n. immunization with CTB, despite the fact that this immunogen given p.o. to human volunteers consistently evoked strong IgA antibody responses in the small intestine (20).

Collectively, the results of this study indicate that at least two distinct subpopulations of ASC precursors arise in the tonsils upon local antigen exposure. One such subpopulation appears to mature within the tonsillar microenvironment, while the other migrates via blood circulation to other locations in the upper aerodigestive mucosa. Such migratory behavior could well be explained by our most recent studies in which we have been able to demonstrate that resident and circulating ASCs induced by i.t. immunization utilize different sets of homing receptors (20a). Whether these two subpopulations originate from the same or distinct B-cell precursor (memory) pool(s) is currently being addressed.

In conclusion, this study demonstrates that human tonsils can serve as powerful inductive sites for local and disseminated antibody responses and are endowed with immunological memory. However, constraints imposed by the tonsillar microenvironment on the migratory behavior of memory and effector B cells may explain the apparent compartmentalization of humoral immune responses initiated in the upper rather than the lower aerodigestive tract. Such a notion should have important bearing on strategies for inducing specific immune protection against upper respiratory infections.

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