SPECIAL FEATURES OF THE PRIMING PROCESS FOR A SECRETORY IgA RESPONSE

B Cell Priming with Cholera Toxin*

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Immunization by the gastrointestinal or respiratory mucosal routes is usually more effective at stimulating a secretory IgA response than parenteral delivery of antigen (1–3). Secretory antibodies generated in gastrointestinal or respiratory mucosa can confer protection against pathogens impinging on adjacent mucosal surfaces (3, 4). Nonreplicating antigens have traditionally been viewed as ineffective in priming for secretory immune responses when given either orally (5, 6) or parenterally (7, 8); however, recent studies using cholera toxin in rats (9, 10), dogs (11, 12), and mice (13, 14) have clearly demonstrated priming by this antigen for an intestinal secretory response when administered by oral, intraduodenal, and even parenteral routes. Priming was scored by enumerating toxoid-binding IgA plasma cells appearing in the gut lamina propria, after intestinal challenge with toxin (9–14). The density of such cells in the intestine correlates with protection against the toxic effects of the enterically delivered antigen (9, 12, 14). This priming process, which results in the generation of appreciable numbers of antigen-specific IgA plasmablasts in gut lamina propria after secondary toxin challenge, probably includes several events: (*a*) antigen-stimulated expansion and differentiation of a specific B cell subpopulation; (*b*) generation of specific helper T cells necessary for the maturation of plasmablasts and the T-dependent expression of IgA antibodies; and (*c*) the in vivo redistribution of antigen-specific B and T cells. We have adapted the rat model of priming for a gut mucosal IgA response against cholera toxin to the inbred mouse in order to resolve these various aspects of priming by taking advantage of the procedures available for analyzing individual murine B cells (15).

Peyer's patches (PP)[1], compared with lymph nodes or spleen, naturally contain a higher frequency of precursors for IgA plasma cells of certain specificities, but not of others (16, 17). However, it is not known where IgA-committed B cells originate or whether the natural antigen load in the gut affects their distribution. Because cholera toxin is not usually an environmental antigen, its administration could permit a controlled re-enactment of the natural priming process that commensal organisms

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[1] *Abbreviations used in this paper:* ACC, antitoxoid-containing cells; CFA, complete Freund's adjuvant; cIg^+, cytoplasmic immunoglobulin positive; MLN, mesenteric lymph node; PBS, phosphate-buffered saline; PP, Peyer's patches; RIA, radioimmunoassay.

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achieve by colonization in the neonate (17). The present report compares mucosal with parenteral routes of cholera toxin/toxoid[2] delivery for stimulation of antigen-specific B cells found in PP and in other lymphoid tissues.

After a secondary toxin challenge in the gut, the PP contribute to the IgA plasmablasts seen in thoracic duct lymph and then in intestinal lamina propria (18). Direct priming of PP cells *in situ* after initial gut mucosal administration of toxin would explain this contribution to the response, but observations in the rat model indicate that intraperitoneal injection of antigen is also effective at priming for the expression of IgA plasmablasts in response to intestinal challenge. The mechanism by which parenteral delivery of antigen affects mucosal lymphoid tissue is unclear, and the general observation that immunization by the mucosal route usually favors the lgA response remains unexplained. We have therefore examined that part of the priming process by cholera toxin/toxoid reflected by quantitative and qualitative changes in the B cell population.

This paper shows that a primary exposure of mice to cholera toxin/toxoid by either the gut mucosal or parenteral route results in a marked increase in antigen-sensitive B cells with secondary characteristics in PP and elsewhere. These cells generate clones in vitro that express IgG and/or IgA isotypes. Mucosal exposure favors higher frequencies of secondary cells committed to the exclusive generation of daughters expressing IgA. Such precursors of clones producing only IgA are more prevalent in PP after mucosal stimulation but exist in significant numbers at distant sites as well. The observations are consistent with our model for B cell development (19, 20), in which permanent commitment of a B cell line to IgA expression is dependent on cell division and is favored when the proliferative stimulus is given in mucosal follicles.

Materials and Methods

Animals. BALB/cJ male mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Mice were immunized at 5–8 wk of age.

Antigens and Immunization Methods. Purified *Vibrio cholera* enterotoxin, lots OZ3052 and EZ3566, was purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. Glutaraldehyde-inactivated cholera toxoid vaccine, Wyeth lot 20201, was the kind gift of Dr. Carl Miller, National Institute of Allergy and Infectious Diseases.

Intraduodenal priming was accomplished by direct injection of 10 μg of purified toxin, in 0.2% gelatin/phosphate-buffered saline (PBS), at the most proximal end of the small intestine after a small laporotomy. Intraperitoneal immunizations used 100 μg toxoid in gelatin/PBS with complete Freund's adjuvant (CFA).

Lamina Propria Plasma Cell Scoring. Single-cell suspensions of intestinal lamina propria were prepared according to a previously published method (21). In some cases, contaminating epithelial cells were removed by separation on a Percoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) gradient by a modification of the technique of Curman et al. (22). The intestinal cell preparation was suspended in 33% isotonic Percoll in PBS, and spun at 17,000 *g* for 30 min at 4°C. Epithelial cells appear in the top third of the self-formed gradient, whereas viable lymphoid cells can be isolated from the bottom third.

Thick smears of these lymphoid cells were fixed in 95% ethanol and stained for antitoxoid containing plasma cells (ACC), using the fluorescent sandwich technique developed by Pierce et al. (9) for staining rat tissues. The toxoid solution for the first step and the fluorescein-labeled rabbit antitoxoid were the kind gifts of Dr. Nathaniel Pierce, The Johns Hopkins University

[2] Cholera toxin/toxoid is used to denote the toxin antigen or its cross-reactive, but nontoxic glutaraldehyde crosslinked toxoid. Toxin was used only for direct intestinal injection; toxoid was used for all other in vivo and in vitro applications.

School of Medicine, Baltimore, Md. A rhodamine-conjugated rabbit antitoxoid was prepared in our laboratory.

Enumeration of Antigen-sensitive Cells. At various times after immunization, toxoid-sensitive B cells in various tissues were enumerated using a modification of the Klinman clonal precursor assay (15). The tissue to be scored was dispersed into single cells and injected at limiting dilution into the tail vein of lethally irradiated (1,500 rad), toxoid-primed scoring recipients. After 16–24 h, the recipients' spleens were removed and diced into ~50 fragments. These fragments were distributed individually among the wells of a microtiter plate, and challenged in culture for 4 d with purified cholera toxoid at a 10^{-7}–10^{-8} M concentration in Dulbecco's modified Eagle's minimal essential medium with 10% agamma horse serum (both from Grand Island Biological Co., Grand Island, N. Y.). The fragments were then washed free of antigen, and maintained in organ culture for an additional 11 d with regular changes of culture medium. Culture fluids were then assayed for antitoxoid antibodies by radioimmunoassay (RIA). The supernatant fluids collected from an individual positive fragment were pooled and used to determine the amount and isotype of antitoxoid antibody produced by the clone of cells contained in that fragment.

RIA. A solid-phase RIA was developed for scoring antitoxoid antibodies in splenic fragment culture fluids. Bromoacetylcellulose (1 g wet wt) was derivatized with 360 mg each of diaminohexane and ϵ-aminocaproic acid at pH 8.9, followed by extensive washing. This aminohexyl cellulose derivative was then added to 5 mg of purified cholera toxoid that had been activated wwih 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Story Chemical Corp., Muskegon, Mich.). Coupling proceeded at room temperature for 8 h, followed by extensive washing to remove noncovalently bound toxoid. This solid support was then used in a standard RIA procedure (23) with ^{125}I-labeled rabbit anti-mouse Fab or rabbit anti-mouse isotype scoring reagents.

Results

Mucosal or Parenteral Stimulation with Antigen Can Prime for an Intestinal Plasma Cell Response to Subsequent Intraduodenal Challenge with Toxin. It has been demonstrated (14) that intestinal ACC responses in the mouse require at least two oral challenges with cholera toxin, whereas as many as seven intravenous challenges result in about one-third the density of ACC in the gut. It was therefore of interest to determine whether both priming procedures we planned to analyze in the mouse did in fact result in a toxoid-binding plasma cell response in gut lamina propria after intraluminal challenge with toxin. Table I shows that both intraperitoneal and intraduodenal priming result in similar ACC frequencies (0.3 and 0.25%, respectively, of total lymphoid cells) at 5 d after a secondary mucosal challenge with toxin.

However, the secondary responses in the gut resulting from priming with antigen

TABLE I

Plasma Cell Populations in Lamina Propria 5 d after Toxin Challenge

	Priming, 14 d before challenge	
	Toxoid/CFA i.p.*	Toxin i.d.‡
(cIg$^+$/total mononuclear cells) × 100%	4	2
(ACC/total mononuclear cells) × 100%	0.3	0.25
(ACC/cIg$^+$) × 100%	8	12
(cIgA$^+$/ACC) × 100%	20	90
(cIgM$^+$/ACC) × 100%	5	0–3
(cIgG$^+$/ACC) × 100%	80	10

* Intraperitoneal.

‡ Intraduodenal.

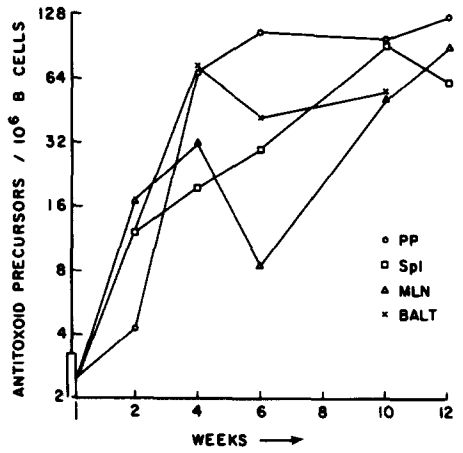

FIG. 1. Enumeration of antitoxoid clonal precursors at various times after intraduodenal priming (↑) with 10 μg cholera toxin. All points represent at least 50 × 10^6 cells scored. Spl, spleen; BALT, bronchus-associated lymphoid tissue.

by the two different routes are dissimilar in their isotype characteristics. Whereas 90% of the antigen-specific plasma cells in intraduodenally primed and challenged mice contain cytoplasmic IgA, only 20% of the ACC from intraperitoneally primed mice contain IgA, and the remainder contain mostly IgG isotypes. This observation supports the general finding that mucosal priming is more efficacious for generating secretory IgA responses (1–4), but the basis for this disparity in the primed cell types resulting from the different routes of immunization remains unclear. To analyze these differences, the effects of priming on the B cell populations found in various lymphoid tissues were investigated.

Intraduodenal Priming with Cholera Toxin Increases the Frequency of Antigen-sensitive Cells in PP and in Distant Lymphoid Tissues. The frequency of toxoid-reactive B cells in normal young adult mouse tissue was first ascertained to serve as a base-line value against which any increase in frequency could be judged. Such an increase in frequency of antigen-sensitive cells can be considered a quantitative index of B cell priming. Fig. 1 shows that all tissues analyzed by the clonal precursor method from unprimed mice (day 0) show frequencies of toxoid-sensitive cells between 1 and 3 per 10^6 B cells. Previous studies (16, 17) have shown that frequencies of this order are found in germ-free or neonatal animals for B cells reactive with common bacterial determinants such as $\beta_2 \rightarrow 1$ fructosyl or phosphocholine, whereas conventional young adult mice, presumably after enteric bacterial association, show frequencies elevated by at least an order of magnitude. Thus, our results suggest that BALB/c mice are naturally in an unprimed state for specificities associated with toxin/toxoid.

Next, the resultant levels of B cell priming at different lymphoid sites were compared after mucosal administration of the antigen. Lymphoid tissues were excised from groups of mice (5–10 per group) at various times after a single intraduodenal challenge with 10 μg cholera toxin, and each tissue type was individually assayed by the clonal precursor method. Fig. 1 shows that 2 wk after mucosal priming, the frequency of toxoid-reactive B cells in PP increased approximately fivefold. Furthermore, the frequency continued to increase at 4 wk and 6 wk postpriming, and the elevated frequency was maintained in the PP even at 12 wk, the latest time tested.

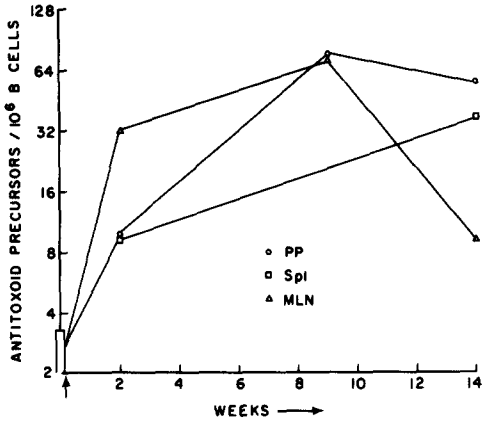

FIG. 2. Enumeration of antitoxoid clonal precursors at various times after intraperitoneal priming (↑) with 100 μg cholera toxoid in CFA. All points represent at least 50 × 10^6 cells scored. Spl, spleen.

TABLE II

Isotype Profile of PP-derived Antitoxoid Clones

Clonal product	B cell donors						
	Week after i.d. priming*					Week after i.p. priming‡	
	0	2	4	6	12	2	12
	Antitoxoid clones expressing product						
	%						
μ only	5	0	0	0	0	0	0
Some μ	75	4	11	15	75	61	5
γ ± α, no μ	10	45	55	62	8	28	90
Some α	20	77	72	85	100	50	42
α only	10	50	33	23	17	11	10
Number of clones analyzed	20	22	18	13	12	18	19

* 10 μg purified cholera toxin injected intraduodenally (i.d.).
‡ 100 μg cholera toxoid injected intraperitoneally (i.p.) with CFA.

This antigen-driven expansion of clonal precursors was also reflected in tissues distant from the site of antigen application. A single intestinal dose of purified cholera toxin caused an increase of reactive B cells at distant mucosal sites of the respiratory tract, as well as in mesenteric lymph node (MLN) and spleen (Fig. 1). Again, the elevated frequencies of antigen-sensitive cells at these distant sites persisted for at least 12 wk after the intestinal challenge, remaining 30- to 100-fold above the background frequencies of unimmunized mice.

Comparison of Intraduodenal and Parenteral Priming of Mucosal and Nonmucosal B Cell Populations. We next examined the expansion of toxoid-sensitive B cell pools in various tissues after parenteral priming. The increase in toxoid-specific clonal precursors after intraperitoneal immunization was comparable to that resulting from mucosal priming (Fig. 2). This increase occurs in mucosal follicles, lymph nodes, and spleen. In particular, the PP show a dramatic elevation in toxoid-sensitive B cells

above the background frequencies in unprimed mice. The elevated frequencies resulting from intraperitoneal priming, however, did not reach as high a level as after intraduodenal priming. By 12–14 wk postpriming, frequencies in intraperitoneally primed mice were ~50% or less of these values in the same tissue from intraduodenally primed mice.

Isotype Potential of Secondary PP Cells Depends on the Route of Immunization. The clonal precursor assay allows not only the enumeration of antigen-sensitive precursors, but also an analysis of the isotype potential of their clonal progeny expressed over 8–10 generations (15). Antitoxoid-producing clones derived from donor tissues after priming by intraduodenal and intraperitoneal routes were therefore analyzed for their isotype profiles to determine whether priming route correlated with isotype potential.

Table II classifies the antitoxoid clones from PP tissues of unprimed mice. Naive animals showed a population profile for clones typical of primary B cells, with a predominance of clones (75%) producing IgM, mostly in combination with other isotypes. A low percentage of antitoxoid clones (10%) from these unprimed mice already displayed a commitment to exclusive IgA production. Shortly after intraduodenal priming, the isotype potential of antitoxoid clonal precursors in the PP shifted to a more secondary character. Less than 5% of the clones analyzed produced any IgM, expression of IgG isotypes was increased more than fourfold, and more than three-quarters of all clones produced some IgA. The most striking feature of these clones is that half of them expressed IgA exclusively. This class of IgA-committed clonal precursors waned at later times after priming, but remained elevated above the unprimed level even at 12 wk after immunization. The overall incidence of IgA expression by clones from the primed cells remained high at all times tested. A depressed frequency of clones expressing IgM was seen immediately after priming and persisted for 6 wk. The return of antigen-sensitive cells giving clones expressing IgM was seen only after 12 wk.

Although a decrease in clones expressing IgM from PP cells was also seen after parenteral priming, antigen administration by this route resulted in less substantial

TABLE III

Isotype Profile of Spleen-derived Antitoxoid Clones

Clonal product	B cell donors					
	Week after i.d. priming*				Week after i.p. priming‡	
	0	2–4	6	12	2	12
	Antitoxoid clones expressing product					
	%					
μ only	10	14	9	9	19	6
Some μ	58	38	42	30	75	12
$\gamma \pm \alpha$, no μ	36	23	39	52	25	82
Some α	21	62	69	56	38	18
α only	5	38	18	17	0	0
Number of clones analyzed	19	13	33	23	16	17

* Intraduodenal.

‡ Intraperitoneal.

increases in any IgA expression. Instead, PP clones from such donors showed an increased expression of IgG isotypes. The secondary character which intraperitoneal priming imparted to the specific B cell population, even to those B cells recovered from mucosal follicles, was manifested as increased potential for IgG production, rather than the commitment to exclusive IgA expression generated by mucosal priming. Specifically, the route of priming was a more significant factor than tissue residence in determining secondary B cell isotype potential.

Splenic Cells Develop Different Isotype Potentials Depending on the Site of Antigen Administration. Table III illustrates the isotype potential of spleen-derived clones from unprimed and primed mice. Mucosally applied antigen generated a population of toxoid-specific clonal precursors in the spleen with a threefold higher frequency of IgA production as compared with that of unprimed splenic tissue. In addition, the percentage of splenic clones committed to exclusive IgA production increased substantially at early times after priming and remained elevated compared with unprimed tissue even at later times.

Parenteral priming did not generate a similar population of splenic cells committed to exclusive IgA expression at any time tested, nor did it increase the total frequency of IgA production by resultant clones to a level comparable with mucosal priming. The secondary character of parenterally primed splenic precursors was manifested as an elevated frequency of cells giving rise to IgG-producing clones seen at later times (3 mo) after priming. Thus, although intraperitoneal priming does develop B cells with secondary characteristics in both spleen and PP, this process occurs more slowly than after mucosal priming, and the potential to express non-IgM isotypes is qualitatively different.

Discussion

The purpose of the present work was to analyze the effects of different priming routes on antigen-specific B lymphocyte populations of mucosal and nonmucosal lymphoid tissues. An assay was used that permits the enumeration of antigen-sensitive cells and analysis of their isotype potential over 8–10 generations (15). Antigen-specific clonal expansion of B cells is a well-established feature of all priming processes for humoral immunity, but the nature of B cell priming in secretory immunity is poorly understood. We therefore chose to examine the antigen-specific response to cholera toxin because it has been found to prime effectively for a secondary intestinal IgA plasma cell response when administered either enterically or parenterally (9–14). Priming by the mucosal, but not the systemic, route requires that the ganglioside-binding activity of the toxin be intact, but this probably relates to the resultant efficiency of transport across mucosal surfaces such as the dome epithelium, rather than to effects on membrane permeability or any other toxic effect (10). We have found that both enteric and parenteral antigen administration prime for a secondary intestinal plasma cell response in the mouse similar to that observed in the rat. However, the mouse shows a distinction in plasma cell isotype profiles engendered by the two methods of priming, whereas this distinction is not as evident in the rat (9).

Using the Klinman clonal precursor assay, we have elucidated several aspects of mucosal priming in the mouse. Both enteric and parenteral antigen were observed to increase markedly the frequency of toxoid-sensitive cells in all lymphoid tissues, and these resultant cells were predominantly secondary in nature, generating clones that

express IgG and/or IgA but not IgM. There are two crucial aspects of this disseminated priming. First, both priming routes generate secondary cells whose progeny may express IgA. This indicates that either route, mucosal or parenteral, could include a mucosal IgA component as part of the overall response to secondary exposure of the gut to toxin/toxoid. Second, PP benefit from the clonal expansion and dissemination of toxoid-sensitive cells, regardless of the priming route, indicating that parenteral priming can provide the mucosal system with secondary memory B cells. This is consistent with previous findings by Husband and Gowans (18), which indicated that ACC appearing in the thoracic duct lymph of parenterally primed rats after an intestinal challenge were of recent PP, and not MLN, origin. However, our studies do point to the possible contribution by MLN and spleen of antitoxoid IgA plasma cell precursors, regardless of priming route.

It should be noted that the clonal precursor assay has certain limitations for studying the overall in vivo priming process for mucosal immunity. It can distinguish the generation, differentiation, and dissemination of primed memory B cells from that of their activated (effector) progeny plasma cells, but it cannot alone definitely predict the timing, magnitude, or site of a secretory immune response in vivo. We can evaluate the full isotype potential of B cells in a particular tissue, but the useful expression of that potential relies on a multitude of factors also influenced by the priming process, such as antigen encounter, plasmablast migration and lodging, and the balance of T-dependent help or suppression at sites of antigen encounter and plasma cell maturation. However, our investigation of mucosal priming shows that the predictive value of the clonal precursor assay is remarkably good. We determined the isotype profile of an intestinal response by two methods. First, the antigen-specific plasma cells in gut lamina propria that appear after toxin challenge in primed mice were scored with fluorescent anti-isotype stains. Second, similarly primed mice were scored by the clonal precursor assay for memory B cells and their isotype potential. Both techniques demonstrate a preponderance of IgA expression, in lamina propria plasma cells and in antigen-sensitive precursors residing in the PP, subsequent to mucosal priming. Furthermore, the increased expression of IgG after parenteral priming is also evidenced by both techniques.

This correspondence between B cell potential assessed in vitro and the actual in vivo expression of different isotypes of plasma cells permits definition of a special feature of priming for a mucosal immune response. The most effective priming for secretory immunity should result in a high frequency of B cells committed to generating clones expressing IgA exclusively. The recirculation of such cells and their lodging in mucosal follicles at surfaces exposed to secondary pathogenic insult would insure the most rapid and efficient source of secretory IgA antibodies. Clearly, mucosally applied antigen is more effective than parenteral antigen at generating IgA-committed cells, and our results indicate that these cells are disseminated beyond the PP, where they initially encountered antigen, to MLN and spleen as well as to distant mucosal follicles. We believe that these IgA-committed cells which are found in the MLN and spleen after mucosal priming originated in the PP and migrated to distant sites after antigenic stimulation. The alternative, that they originated in the MLN or spleen and proliferated there in response to antigen leaked from the intestine and encountered at these nonenteric sites, is unlikely because systemically introduced

toxin in this dose range has never been shown to prime successfully for secretory IgA responses either in mice (7, 14) or in rats (24).

Thus the route of priming is more influential than the ultimate tissue residence in determining the isotype profile of secondary B cells. Mucosal priming establishes a differential in the concentration of IgA-committed B cells, so that PP contain more than spleen. IgA-committed cells are also generated by parenteral priming, but in much lower frequencies. We believe these observations indicate focal priming after mucosal stimulation and that cells committed to IgA increase in PP mainly through clonal expansion at that site, rather than by accumulation of IgA-committed cells which may be generated elsewhere.

If local priming promotes the generation of IgA-committed cells in the PP, then the patch must provide a unique environment which allows this specialized differentiation. We have previously proposed that V_H-gene translocations within a B cell line follow a division-dependent vectorial process with a V_H-C_α juxtaposition as the terminal consequence (19, 20). Continual antigen-driven division in the absence of clonal attenuation through maturation to plasma cells should therefore increase the probability of arriving at a V_H-C_α recombination and concomitant IgA commitment. One prediction this model makes is that secondary cells generated by parenteral priming, which normally yield clones expressing IgG ± IgA, could become committed to exclusive IgA expression as a result of further divisions in the PP. Ultimately, the special features of the patch milieu that abrogate plasma cell maturation must also be defined. These might include unique subsets of antigen-presenting cells and/or T cells (25–27).

Although B cells committed to IgA expression may be generated in the PP, effective mucosal immunity requires the dissemination of their plasmablast progeny. The influence of antigen on the distribution of large lymphocytes in the mucosal system has been investigated by many workers. Selective lodging of such cells is relatively independent of bacterial or other enteric antigens as suggested from studies using embryonic and neonatal gut explants (28, 29). However, an altered distribution of antigen-specific plasma cells has been demonstrated in response to localized antigen (9, 18). Both hormonal and tissue-specific influences have also been cited as influencing lymphoblast migration in cases where antigen gradients were unlikely to serve as a determining influence for lodging (30–33). We find that the antigen-specific memory B cells, which are the precursors for these plasmablasts and plasma cells, appear to distribute throughout the lymphoid system in an antigen-independent fashion. Furthermore, this dissemination yields a persistent memory population at sites distant from the antigenic challenge, resulting in cross-priming among mucosal follicles.

Summary

Administration of cholera toxin/toxoid by either intraduodenal or parenteral routes increases the frequency of antigen-sensitive B cells in Peyer's patches (PP) and in distant lymphoid tissues > 50-fold. The special feature of mucosal priming with toxin is its unique effectiveness at generating secondary B cells, whose progeny express IgA exclusively, and such cells appear in highest frequency in PP and in appreciable numbers in spleen. Thus, this deliberate intraduodenal immunization seems to mimic the natural priming process induced by enteric bacterial colonization, which we have postulated to account for the high frequencies of IgA-committed cells specific for

bacterial determinants in the PP of conventionally reared mice. Furthermore, as a result of intraduodenal immunization, antigen-specific memory B cells are disseminated to sites distant from that of antigen application, including the lymphoid follicles associated with the respiratory mucosa. Direct antigenic stimulation of cells in the PP therefore results in effective cross-priming among mucosal and systemic sites through division, differentiation, and dissemination of antigen-sensitive secondary B cells.

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