IgA Class Switch Occurs in the Organized Nasopharynx- and Gut-Associated Lymphoid Tissue, but Not in the Diffuse Lamina Propria of Airways and Gut

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IgA Class Switch Occurs in the Organized Nasopharynx- and Gut-Associated Lymphoid Tissue, but Not in the Diffuse Lamina Propria of Airways and Gut

Takashi Shikina,*† Takachika Hiroi,*‡ Kohichi Iwatanai,* Myyoung Ho Jang,* Satoshi Fukuyama,*¶ Manabu Tamura,† Takeshi Kubo,‡ Hiromichi Ishikawa,§ and Hiroshi Kiyono2*‡¶

Secretory IgA plays a crucial role in the host immune response as a first line of defense. A recent demonstration of in situ IgA class switching in intestinal lamina propria provided an opportunity to reconsider the model for the homing of IgA-committed B cells characterized by distinctive trafficking patterns to effector sites. Those effector sites depend on the organized mucosa-associated lymphoid tissues as their site of induction. In this report we show the preferential presence of IgM⁺B220⁺ and IgA⁺B220⁺ cells belonging to pre- and post-IgA isotype class-switched cells in the organized mucosa-associated lymphoid tissues, such as nasopharynx-associated lymphoid tissues, isolated lymphoid follicles, and Peyer’s patches, and the defect of those populations in the diffuse effector sites, such as the nasal passage and intestinal lamina propria. Consistent with these findings, the expressions of a series of IgA isotype class switch recombination-related molecules, including activation-induced cytidine deaminase, Iα-Cμ circle transcripts, and Iα-Cμ μ circle transcripts, were selectively detected in these organized mucosa-associated lymphoid structures, but not in the diffuse mucosal effector sites. Taken together, these findings suggest that IgA isotype class switching occurs only in the organized mucosa-associated lymphoid organs (e.g., nasopharynx-associated lymphoid tissues, isolated lymphoid follicles, and Peyer’s patches), but not in the diffuse effector tissues of the upper respiratory and gastrointestinal tracts. The Journal of Immunology, 2004, 172: 6259–6264.

The mucosal immune system provides the first line of defense against the ingress of microbial pathogens during the physiological processes of inhalation and ingestion. One of the major components of the mucosal immune system is secretory IgA (S-IgA), which is produced by the mucosal interaction of epithelial cells, IgA-committed B cells, and Th cells in the respiratory and gastrointestinal tracts (1, 2). Thus, mucosal effector sites such as the lamina propria of the upper respiratory and intestinal tract contain high numbers of IgA blast and plasma cells derived from postswitched IgA⁺ B cells. IgA-committed B cells that have undergone μ to α isotype class switching in nasopharynx-associated lymphoid tissue (NALT), Peyer’s patches (PPs), and other mucosal inductive lymphoid organs are generally believed to migrate to diffuse mucosal effector tissues, including the nasal passage (NP) and intestinal lamina propria (i-LP) (3, 4). PPs show a high frequency of IgM⁺B220⁺ B cells, which are a prerequisite for isotype class switching to IgA⁺B220⁺ B cells after antigenic or mitogenic stimulation in the presence of the isotype switch-inducing cytokine TGF-β (1, 5–8). PPs are an example of organized gut-associated lymphoid tissue (GALT) with germinal centers (GCs) and are thus considered to be a major site for the μ to α class switch recombination (CSR) involving activation-induced cytidine deaminase (AID), Iα-Cμ μ circle transcripts (αCTs), and Iα-α transcripts (9, 10). Recent results, generated from analysis of AID-deficient mice, however, have suggested another pathway for the development of intestinal IgA plasma cells (11). IgA class switching was shown to occur in i-LP without the involvement of PPs or other organized mucosa-associated lymphoid tissues (MALT) containing GCs. In this study, IgM⁺B220⁺ B cells in i-LP switched to IgA⁺ B cells under the influence of TGF-β derived from i-LP stromal cells (11). These findings suggest that i-LP could have the immunological function of both effector and inductive sites. However, the concerns with that study were that the researchers did not have control of the compartment from which the B cells were harvested, either the diffuse lamina propria or isolated lymphoid follicles (ILFs) embedded in the lamina propria, due to the nature of AID-deficient mice. Because AID⁻/⁻ mice exhibited enormous hyperplasia of ILFs, there was little of the diffuse lamina propria region left (11).

We recently identified ILFs as part of the organized GALT in mouse small intestine (12). ILFs are comprised of a single B cell

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Abbreviations used in this paper: S-IgA, secretory IgA; AID, activation-induced cytidine deaminase; CSR, classswitch recombination; CT, cholera toxin; αCT; Iα-Cμ μ circle transcript; GALT, gut-associated lymphoid tissue; GC, germinal center; ILF, isolated lymphoid follicle; i-LP, intestinal lamina propria; MC, mononuclear cell; MLN, mesenteric lymph node; NALT, nasopharynx-associated lymphoid tissue; NP, nasal passage; PP, Peyer’s patch.

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follicle with GCs overlaid by a follicle-associated epithelium containing Ag up-taking M cells that are similar to the follicle-associated epithelium of PPs. The presence of ILF tissue could provide additional mucosal inductive sites for the generation of IgA-committed B cells in the gastrointestinal tract (13). In fact, looking at the data overall, it is not possible to exclude the possibility that ordinary preparations of i-LP samples may contain IgM/B220 and IgA/B220 B cell fractions undergoing in situ class switching inside the GCs of these newly discovered murine ILFs (12).

For the upper respiratory tract, NALT has been shown to contain all the necessary molecular and cellular environments for the initiation of IgA B cell responses (2, 14, 15). NALT has also been identified as a region in which both IgA-committed B cells and memory-type IgA B cells are generated (15). Nasal immunization has been shown to effectively induce Ag-specific IgA Ab responses via NALT for the upper respiratory tract, including the NP (2, 16). Thus, in the respiratory tract, NALT, with its inductive sites, and NP, with its effector sites, are considered to be part of the IgA-committed B cell-homing pathway analogous with PPs and i-LP in the gastrointestinal tract.

In this study, using the molecular and cellular analyses of transcription as well as immunocytochemistry and immunohistochemistry, NALT and GALT, including ILFs and PPs as well as NP and i-LP, were examined as examples of organized inductive and diffuse effector tissues, respectively. In this paper we report evidence that organized MALT, but not the diffuse lamina propria of the respiratory and intestinal tracts, play an essential role in the generation of IgA-committed B cells, especially in the μ to α isotype class switching for the mucosal immune system.

Materials and Methods

Mice

BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and used at 6–12 wk of age. In some experiments mice were nasally immunized with...
1 μg of cholera toxin (CT; Sigma-Aldrich, St. Louis, MO), a potent immunogen with strong adjuvanticity (17), once a week for 3 consecutive wk (16).

**Cell preparation**

Mononuclear cells (MCs) were isolated from spleen, PPs, NP, and NALT as described previously (16, 18). MCs from spleen and NALT were obtained using a mechanical dissociation procedure (16, 18). MCs from PPs and NP were prepared using the enzymatic dissociation protocol with collagenase D (Roche, Mannheim, Germany) (16, 18). MCs from ILFs were prepared as described previously (12). In brief, the small intestine was opened longitudinally along the mesenteric wall. After removal of mucus and feces, a 3-cm intestinal fragment was pasted on a culture dish. Under a transillumination stereomicroscope (TH3; Olympus, Tokyo, Japan), a tiny fragment of the small intestine containing a single ILF was amputated by a sharpened needle. After removal of PPs and ILFs, i-LP lymphocytes were isolated from the small intestine by the enzymatic dissociation procedure with collagenase D (Roche) (18).

**Flow cytometry**

FITC-conjugated anti-mouse IgA and IgM, and R-PE-conjugated anti-B220 were used for staining (BD PharMingen, San Jose, CA) (18). MCs isolated from different tissues were preincubated with Fc Block (2 μg/ml; BD PharMingen) before fluorochrome-conjugated mAbs. MCs were then incubated with optimal concentrations of FITC-conjugated anti-mouse IgA (2 μg/ml) or IgM (2 μg/ml) together with PE-conjugated anti-B220 (2 μg/ml). Flow cytometric analysis was then performed using FACSCalibur (BD Biosciences, San Jose, CA) (18).

**Immunohistochemical analysis**

Vertically oriented sections of the small intestine that included PPs and/or ILFs as well as NP and NALT were prepared as previously described (2, 12). Briefly, the small intestine was longitudinally opened along the mesenteric wall, rolled up, and frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen. Serial frozen sections were then incubated with FITC-conjugated anti-IgA (2 μg/ml) or FITC-conjugated anti-IgM (2 μg/ml) and PE-conjugated anti-B220/CD45R (2 μg/ml; all from BD PharMingen) for 1 h at room temperature. The slides were then examined using confocal microscopy (Bio-Rad, Hercules, CA). After confocal fluorescence microscopic analysis, the sections were counterstained with H&E. The sections used for examination were prepared from at least three individual mice.

**FIGURE 2.** Selective localization of IgM⁺B220⁺ cells in NALT, but not in NP. A, MCs isolated from NALT and NP were stained with a panel of mAbs specific for IgM, IgA, and B220. B, Consecutive tissue sections were stained with FITC-conjugated anti-IgA (a and d), anti-IgM (b and e), and PE-conjugated anti-B220 (a, b, d, and e). Samples of each tissue were also stained with HE (c and f). Cells expressing IgA or IgM (green) as well as those expressing B220 (red) were found only in NALT (a and b). The numbers of double-positive cells (yellow) of IgA or IgM and B220 were negligible (d and e), although IgA⁺B220⁺ blasts and plasma cells were plentiful (d). C, IgA⁺B220⁺ B cells increased in NALT after nasal immunization with CT. MCs isolated from NALT were stained with a panel of mAbs specific for IgM, IgA, and B220. Consecutive tissue sections were stained with FITC-conjugated anti-IgM and PE-conjugated anti-B220 (a) or FITC-conjugated anti-IgA and PE-conjugated anti-B220 (b). Higher magnification of sections a and b are shown in c and d, respectively. Double-positive cells (yellow) for IgA (green) and B220 (red) were increased in NALT after nasal immunization with CT (0.8–2.9%). In contrast, double-positive cells (yellow) for IgM (green) and B220 (red) were found in both naive and immunized NALT (B-b and C-a or C-c). These sections were analyzed by confocal microscopy using ×35 (B, C-a, and C-b) and ×200 (C-c and C-d) magnifications. Results are representative of three separate experiments.
RT-PCR

Total RNA isolated from mouse tissues was extracted, following the manufacturer’s instructions, using TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared by reverse transcriptase (Invitrogen) with oligo(dT) primer. AID transcripts, αCTs, Iκ-Cα, and β-actin transcripts were amplified as described previously (9–11). The oligonucleotide primers specific for AID transcripts (5′-GGCTGAGTTAGGGTTCATCTCAG-3′ and 5′-GAGG GAGTCCAAAGGATCAGCCTGA-3′), Igα–Cy circle transcripts (5′- CCAGGCATGGTTAGATAGAGATAGGAGGAGCAGAAAGattaAGATAG-3′ and 5′-AATGGTGCCTCTGAGGAAATGATGCTGCCAACTGATGCTGGAATGATGCTAGG-3′), Iκ-Cα transcripts (5′-CTCTGCGCTGCTTATTGTG TG-3′ and 5′-GAGCTGGTGAGGATGTTGCTCAGTTG-3′), and β-actin transcripts (5′-TGGAATCTTGGCCCTCTGATGACC-3′ and 5′-TAAACACGACGC TGATGACCAGCTG-3′) were prepared according to previously described methods (9–11). The experiments were conducted on three separate occasions.

Results

Focal accumulation of IgM⁺B220⁺ and IgA⁺B220⁺ cells in organized GALT structures, but not diffuse effector tissues

To investigate the exact class-switching sites in different parts of the wall of the mouse small intestine, we initially examined and compared the localized presence of IgM⁺B220⁺ B cells, which are considered to be prerequisite for class switching recombination (19, 20). To accomplish this goal, careful separation of the organized lymphoid tissue and the diffuse effector tissue was required. GALT, including PPs and especially ILFs, was carefully identified and removed. After the removal of GALT, but before enzymatic dissociation, the intestine was further re-examined, using microscopic analysis, to ensure and confirm the complete removal of organized lymphoid tissue. Screening for IgM⁺B220⁺ B cells revealed their presence in ILFs and PPs, but their almost complete absence from i-LP (Fig. 2, A and Table I). Furthermore, because IgA⁺B220⁺ B cells are believed to result from recent class switching involving the expression of CSR molecules such as AID and looped-out circular DNA (11), we next screened for the presence of IgA⁺B220⁺ B cells in these different intestinal mucosa-associated tissues. Although FACS analysis revealed the presence of IgA⁺B220⁺ B cells in ILFs and PPs, hardly any were detected in i-LP (Fig. 1A and Table I). Furthermore, the preferential localization of both IgM⁺B220⁺ cells and IgA⁺B220⁺ B cells in the organized lymphoid tissues was also revealed by immunohistochemical analysis using fluorescence confocal microscopy (Fig. 1B). By contrast, both populations were absent in diffuse i-LP regions. Instead, larger numbers of IgA⁺B220⁺ B cells, corresponding to the blast and plasma stages, were found in diffuse effector tissues (Table I). Taken together, these observations suggest that the isotype class switching of B cells from μ- to α-chains occurs selectively in the organized lymphoid structure of ILFs and PPs, but not in the diffuse effector tissues of i-LP of the small intestine.

In the upper respiratory tract, IgM⁺B220⁺ and IgA⁺B220⁺ B cells are present in NALT, but not in NP

In the generation of IgA-committed B cells, NALT has been shown to be as important an inductive site as GALT (1, 15, 16). When similar evaluation of the respiratory mucosal immune system for IgM⁺B220⁺ B cell presence was conducted, the tissue localization pattern was similar to that we described for the intestinal tract. Thus, IgM⁺B220⁺ B cells were preferentially localized in the organized NALT, but were virtually absent from the diffuse effector tissues of the NP (Fig. 2, B-b or B-e, and Table I). In contrast to PPs and ILFs, extremely few IgA⁺B220⁺ B cells were found in NALT (Fig. 2a-c and Table I). Ag stimulation by bacterial flora of the upper respiratory tract is weaker than that by gut flora surrounding GALT, and we hypothesized that this was the reason for the lack of IgA⁺B220⁺ B cells in NALT. To test this hypothesis we performed nasal immunization with CT, which is known to possess a strong antigenticity with potent mucosal adjuvant activity. MCs were isolated from NALT and then analyzed by flow cytometry with fluorochrome-conjugated mAbs anti-μ, anti-α, or PE-conjugated mAb anti-B220. Percentages of the B cell subset indicated in the left column in NALT before and after nasal immunization with CT are shown in each row. The data are presented as the mean ± SD from three separate experiments. slg, surface Ig.

Expression of CSR-associated mRNA observed in the organized MALT structure, but not in diffuse effector tissues

To test for CSR from the μ-chain to the α-chain at the molecular level, three molecular markers, AID, αCTs, and Iκ-Cα, were selected. AID was essential for CSR and completely regulated stimulated B cells undergoing class switching (9–11). αCTs were produced from circular DNA that is looped out and lost after CSR (9–11). Iκ-Cα transcripts were produced from α germine transcripts after looping out of αCTs (9–11). Because the expression of AID and αCTs is strictly up-regulated during and is quickly down-regulated after isotype class switching, such expression is considered to characterize the class switching of B cells from μ to α (10). Meanwhile, Iκ-Cα transcripts were seen to be expressed immediately after completion of IgA-specific CSR (11). To further confirm that IgA isotype class switching occurs in vivo at the organized MALT, we next used RT-PCR analysis to test for the expression of CSR-associated molecules, including AID, αCTs, and Table II ). Influence of nasal CT on the development of IgA⁺ B cells in NALT

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*After nasal immunization with CT, which is known to possess a strong antigenticity with potent mucosal adjuvant activity (17). After nasal immunization, the presence of IgA⁺B220⁺ B cells increased in NALT (Fig. 2C and Table II). The formation of GCs and the accumulation of IgA⁺B220⁺ B cells were also seen in the NALT of these nasally immunized mice (Fig. 2C). These results demonstrate that in the upper respiratory tract, IgA isotype class switching occurs in the organized NALT, but not in the diffuse lamina propria of the NP.

FIGURE 3. Preferential presence of AID, αCT, and μ-Cα transcripts in the organized lymphoid tissues of NALT, PPs, and ILFs, but not in the diffuse effector tissues of NP and i-LP. RT-PCR analysis revealed AID, αCT, and μ-Cα transcripts in NALT, PPs, and ILFs, but not in i-LP and NP. As a negative control for RT-PCR, the total RNA of MCs isolated from spleen of recombinase-activating gene-1(RAG1)⁻/⁻ mice was used. The experiments were conducted on three separate occasions.
and \( \mu\)-\( \gamma \) transcripts, in the organized lymphoid and diffuse effector tissues of the gastrointestinal and respiratory tracts. The results were consistent with our finding of histological localization of IgM \(^{-} \) B220 \(^{-} \) B cells and IgA \(^{-} \) B220 \(^{-} \) B cells (Figs. 1 and 2, and Tables I and II). mRNA expressions of AID, \( \alpha CTs, \) and \( \mu\)-\( \gamma \) transcripts were restricted to the organized MALT, such as ILFs, PPs, and NALT, but were not found in the diffuse effector tissues of i-LP and NP (Fig. 3). These results indicate that IgA isotype class switching selectively occurs in the organized MALT, but not in the diffuse effector tissues.

**Discussion**

PPs have been shown to contain all the cellular and microarchitectural environments (e.g., B cell follicle, including GCs, follicular dendritic cell networks, and interfollicular T cell area) needed for the generation of IgA-committed B cells (1). A large number of IgM \(^{-} \) B220 \(^{-} \) B cells in the GCs of PP follicles express AID and undergo the molecular event of \( \mu \) to \( \alpha \) isotype class switching (9). Results have shown that the incubation of IgM \(^{-} \) IgA \(^{-} \) B cells isolated from PPs together with TGF-\( \beta \) results in the induction of IgA isotype class switching, which leads to the generation of IgM \( \rightarrow \) IgA \(^{-} \) B cells (5–8). After generation in PPs, these post-switched IgA \(^{-} \) B cells (or IgA-committed B cells) migrate to effector tissues, such as i-LP, where they become IgA blast and plasma cells under the influence of IgA-enhancing cytokines IL-5, IL-6, and IL-10 (1). Therefore, it is generally accepted that organized MALT, such as PPs, acting as inductive sites, play a major role in the initiation of the IgA Ab response, while diffuse i-LP tissues provide the effector sites (1). This conjecture was further confirmed by our current results, which show that IgM \(^{-} \) B220 \(^{-} \) B cells capable of undergoing \( \mu \) to \( \alpha \) isotype class switching were exclusively located in PPs and were absent from diffuse i-LP. In contrast, large numbers of IgA blast and plasma cells were found in these diffuse i-LP. Furthermore, evaluation of CSR-associated molecules for the \( \mu \) to \( \alpha \) gene rearrangement revealed that AID, \( \alpha CTs, \) and \( \mu\)-\( \gamma \) transcripts were selectively expressed in PPs with the organized lymphoid structure, but not in the diffuse i-LP.

In addition to testing PPs, we tested ILFs, which we recently characterized as part of the organized GALT on the antimesenteric wall of the mouse small intestine (12). This cell formation is composed of a large B cell area, including GCs. A large fraction of the B cells in ILFs are B-2 cells, similar to those found in PPs (12). In this study our findings further demonstrate that ILFs are a rich source of IgM \(^{-} \) B220 \(^{-} \) B cells that can undergo \( \mu \) to \( \alpha \) CSR. Thus, CSR-associated transcripts of AID, \( \alpha CT, \) and \( \mu\)-\( \gamma \) were present in the mRNA preparation obtained from ILF as well as PP samples. After careful removal of ILFs and PPs, MCs that had been isolated from the diffuse effector tissues of i-LP were evaluated for the IgA-associated CSR-associated transcripts. No AID, \( \alpha CT, \) or \( \mu\)-\( \gamma \) transcripts were found in the diffuse tissue of i-LP. Taken together, these findings indicate that ILFs and PPs, both of which contain B cell follicles with GCs, constitute the organized GALT and behave as key inductive sites for \( \mu \) to \( \alpha \) isotype class switching when IgA-committed B cells are generated.

When we evaluated the presence and population density of IgM \(^{-} \) B cells and/or IgA \(^{-} \) B cells in different histological locations of the upper respiratory tract, accumulations of IgM \(^{-} \) B220 \(^{-} \) B cells were always observed only in the organized MALT such as NALT. These cells were absent in the NP, a representative diffuse effector site for the upper respiratory tract (Fig. 2 and Table I). In addition, AID, \( \alpha CT, \) and \( \mu\)-\( \gamma \) transcripts were expressed in NALT, but not in NP, paralleling expression by ILFs and PPs, but not i-LP, in the intestinal tract (Fig. 3). Previously we have shown that NALT B cells belong to a subset of B-2 cells (18). That study also showed that IgA isotype class switching of B-2 cells in the upper respiratory tract occurs only in the organized MALT, such as NALT, and not in the diffuse lamina propria region of the NP.

Comparisons of NALT and GALT (e.g., ILFs and PPs) for the population density of switched IgA \(^{-} \) B220 \(^{-} \) B cells revealed a dramatic difference. Much higher numbers of IgA \(^{-} \) B220 \(^{-} \) B cells were found in GALT (e.g., 0.5–14.4%; Table I) than in NALT (e.g., 0.2–1.4%; Table I). That finding may be explained by the different microbial environments found in NALT and GALT. In comparison with NALT, GALT structures are situated in a part of the gut where there is an enormous load of microbial Ags and mitogens to continuously stimulate immunocompetent cells located in the organized lymphoid tissue for the mucosal immune system. Thus, administration of CT possessing potent immunogenicity and adjuvanticity via the nasal route resulted in a 3- to 10-fold increase in the population (e.g., 2.9 ± 0.7%; Fig. 2). This increase points to the importance of antigenic stimulation in the initiation of IgA isotype class switching for the generation of IgA-committed B cells in NALT. Elsewhere, we have shown that the tissue organogenesis of NALT is also accelerated by nasal exposure to Ag (21).

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**References**


