Bacteroides Induce Higher IgA Production Than Lactobacillus by Increasing Activation-Induced Cytidine Deaminase Expression in B Cells in Murine Peyer’s Patches

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Received September 1, 2008; Accepted September 29, 2008; Online Publication, February 7, 2009

[doi:10.1271/bbb.80612]

The gut mucosal immune system is crucial in host defense against infection by pathogenic microbacteria and viruses via the production of IgA. Previous studies have shown that intestinal commensal bacteria enhance mucosal IgA production. However, it is poorly understood how these bacteria induce IgA production and which genera of intestinal commensal bacteria induce IgA production effectively. In this study, we compared the immunomodulatory effects of Bacteroides and Lactobacillus on IgA production by Peyer’s patches lymphocytes. IgA production by Peyer’s patches lymphocytes co-cultured with Bacteroides was higher than with Lactobacillus. In addition, the expression of activation-induced cytidine deaminase increased in co-culture with Bacteroides but not with Lactobacillus. We found that intestinal commensal bacteria elicited IgA production. In particular, Bacteroides induced the differentiation of Peyer’s patches B cell into IgA+ B cells by increasing activation-induced cytidine deaminase expression.

Key words: IgA; intestinal commensal bacteria; Bacteroides; Peyer’s patches; activation-induced cytidine deaminase

The intestine is continually exposed to a range of infectious pathogens such as bacteria and viruses, as the mucosa acts as a border between the body and the environment. Intestinal mucosal defenses to these microorganisms are initiated by IgA secretion. While it is well known that in the human intestine there are more than 100 trillion commensal bacteria, these bacteria are not eliminated completely.11 Previous studies with germ-free and conventional (or specific pathogen free) mice have demonstrated that intestinal microorganisms increase the numbers of IgA-secreting plasma cells (IgA-PCs), and then up-regulate IgA secretion,2,3 but there are more than 500 species of bacteria living in the human intestine with symbiotic relationships and it is poorly understood whether the different intestinal commensal bacteria have different effects on IgA production.4 Moreover, the precise mechanism underlying the way these bacteria modulate the intestinal immune system is also yet to be fully elucidated.

Microbial antigens existing in the intestinal lumen are taken up through microfold (M) cells that are located in the epithelial layers of Payer’s patches (PPs) and villi, or are captured by dendritic cells (DCs) putting out tentacles from tight junctions of intestinal epithelial cells.5–7) These antigens are then presented to IgM+ B cells, and IgA class switch recombination (CSR) from μ to α is induced by T cell-dependent or independent pathways through antigen presenting cells such as DCs,5,9) It is known that activation-induced cytidine deaminase (AID) is the enzyme required for Ig gene CSR and somatic hypermutation.10) IgA+ B cells expressing the sphingosin 1-phosphate receptor, chemokine receptors such as CCR9, and adhesion molecules such as the integrin α4β7 traffic from PP to lamina propria, and then IgA+ B cells terminally differentiate to IgA-PCs.11–13) Although Bacteroides are a major intestinal commensal bacteria in the murine intestinal microbiota, their immunological function is yet to be defined. On the other hand, Lactobacillus, one of the predominant microorganisms in the murine microbiota, are utilized as probiotics and have an anticipated regulatory functions in the intestinal environment, such as regulation of allergic hypersensitivity and food allergy.14,15)

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Abbreviations: PP, Peyer’s patch; AID, activation-induced cytidine deaminase; CSR, class switch recombination; DCs, dendritic cells; IgA-PCs, IgA-secreting plasma cells; FCS, fetal calf serum; MLN, mesenteric lymph nodes; FOS, fluctooligosaccharides; IL, interleukin
Materials and Methods

Mice. BALB/c mice were purchased from CLEA Japan (Tokyo) and were used at 10 weeks of age. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the College of Bioresource Sciences, Nihon University.

Bacteria preparation. Three strains of Bacteroidaceae (B7, unidentified; B18, unidentified; and B23, B. acidifaciens type A43),17,18 and three strains of lactobacilli (LA, L. johnsonii 129, originally identified as L. acidophilus; LF, L. reuteri 106, originally identified as L. fermentum; and LM, L. murinus 91, originally identified as L. murinum),17,18 were isolated from murine intestinal commensal bacteria. All the Bacteroides were cultured in Eggert-Gagon (EG) broth without horse blood supplemented with 1/1,000 volume of Hemin solution (0.1% hemin (Tokyo Chemical Industry, Tokyo) in 0.2% NaOH) and 1/1,000 volume of Menadione solution (5 mg/ml of menadione (Sigma Aldrich, St. Louis, MO)) in ethanol), and lactobacilli were cultured in MRS broth (Difco, Franklin Lakes, NJ) for 18–24 h at 37 °C under anaerobic conditions, respectively.21) The cultured bacteria were harvested by centrifugation and washed with PBS. Then the bacterial suspensions were heated at 80 °C for 50 min and washed with PBS. These bacteria were lyophilized and used in vitro.

Cell preparation and culture. PPs lymphocytes were obtained as described previously and cultured at 5 × 10^6 cells/ml with RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml).22) After incubation for 24 h, the PPs lymphocytes were supplemented with lyophilized bacteria suspended in RPMI 1640 with 10% FCS at 10^6/g/ml final concentration (PPs lymphocytes 2.5 × 10^6 cells/ml final concentration) and then co-cultured for 3 or 7 d. We have observed that the concentration of the Bifidobacterium component (10–50 μg/ml) is suitable for the induction of PP immunoreponses in vitro.22) In addition, we confirmed that PPs lymphocytes were induced at the same levels of IgA and of IL-6 production by co-culture with 10 and 100 μg/ml of Bacteroidaceae and lactobacilli respectively, when we compared bacterial concentrations of 1, 10, and 100 μg/ml.

Determination of cytokines and total IgA. The 3d culture supernatants were used in the detection of interleukin (IL)-5 and IL-6 productions. Supernatants from 7 d cultures were assayed for IgA production. IL-5, 6, and IgA were detected by sandwich ELISA, as described previously.23) In IgA detection, we used capture and detection antibodies as follows: goat anti-mouse IgG fragment (Sigma Aldrich) and alkaline phosphatase conjugated goat anti-mouse IgA antibody (Southern Biotech, Birmingham, AL).

Flow cytometric analysis of cell surface markers on PPs lymphocytes. PPs lymphocytes Fc receptors were blocked with CD16/32 (93; eBioscience, San Diego, CA), and then incubated with optimal concentrations of FITC-conjugated anti-mouse IgA (C10-3; BD Biosciences Franklin Lakes, NJ), PE-conjugated anti-mouse Syn-decan-1 (281-2; BD Biosciences), APC-Cy7-conjugated anti-mouse B220 (RA3-6B2; BD Biosciences), and Propidium Iodide (Sigma Aldrich). Flow cytometric analysis was then performed using FACSCanto (BD Biosciences). Analysis was performed using FlowJo ver.8 (Tree Star, Ashland, OR).

Results

Bacteroides induced PPs lymphocytes to produce higher levels of IgA than Lactobacillus in vitro

We examined the immunomodulation of Bacteroides and of Lactobacillus on IgA production by PPs lymphocytes. We determined and compared the total IgA in the supernatants of PPs lymphocytes co-cultured with Bacteroidaceae that B7, B18, and B23, and with lactobacilli that LA, LM and LF for 7 d. The bacterial concentrations of 1, 10, and 100 μg/ml of Bacteroidaceae and lactobacilli were used at 10 weeks of age. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the College of Bioresource Sciences, Nihon University.

After pre-culture at 2 °C for 2 d, total RNAs were isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Complementary DNA was prepared by reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT) primer. Real-time RT-PCR analyses were done in duplicate on a LightCycler 480 (Roche, Mannheim, Germany) with a LightCycler 480 SYBER Green I Master (Roche). To detect AID, primers AID F, 5′-AAATTTCTGTCGGCTAAACCA-3′, and AID R, 5′-CATTCCAGGAGGTGTCTTTC-3′, were used. Cycling was performed under the following conditions: denaturation at 95 °C for 20 s, followed by 40 cycles of PCR (95 °C for 10 s, 63 °C for 10 s, and 72 °C for 4.6 s). To detect GAPDH, primers GAPDH F, 5′-TGAAACGGAAGCTCACTTG-3′, and GAPDH R, 5′-TCCACA-CCTGTGGCCTGTA-3′, were used. Cycling was performed under the following conditions: denaturation at 95 °C for 10 s, followed by 45 cycles of PCR (95 °C for 10 s, 59 °C for 10 s, and 72 °C for 4.4 s). The correct size of the amplification products obtained was confirmed by melting curve analysis. The amount of AID mRNA was normalized to the amount of GAPDH mRNA of the P P B cells (the amount of GAPDH mRNA × the frequency of B220 cells and IgA^+ B220 cells obtained by flow cytometric analysis).

Statistical analysis. Data were expressed as the mean ± SD. Analysis of the data was by one-way ANOVA. In addition, a comparison of the mean of the Bacteroides and the Lactobacillus groups, was performed by Student’s t test. All the data were analyzed using SPSS ver. 13.0 (SPSS, Chicago, IL).

Quantitative analysis of expression of AID mRNA. PPs lymphocytes were cultured for 24 h and then co-cultured with three strains of lactobacilli and Bacteroidaceae for 3 d and used in quantitative real-time RT-PCR. Total RNAs were isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Complementary DNA was prepared by reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo(dT) primer. Real-time RT-PCR analyses were done in duplicate on a LightCycler 480 (Roche, Mannheim, Germany) with a LightCycler 480 SYBER Green I Master (Roche). To detect AID, primers AID F, 5′-AAATTTCTGTCGGCTAAACCA-3′, and AID R, 5′-CATTCCAGGAGGTGTCTTTC-3′, were used. Cycling was performed under the following conditions: denaturation at 95 °C for 20 s, followed by 40 cycles of PCR (95 °C for 10 s, 63 °C for 10 s, and 72 °C for 4.6 s). To detect GAPDH, primers GAPDH F, 5′-TGAAACGGAAGCTCACTTG-3′, and GAPDH R, 5′-TCCACA-CCTGTGGCCTGTA-3′, were used. Cycling was performed under the following conditions: denaturation at 95 °C for 10 s, followed by 45 cycles of PCR (95 °C for 10 s, 59 °C for 10 s, and 72 °C for 4.4 s). The correct size of the amplification products obtained was confirmed by melting curve analysis. The amount of AID mRNA was normalized to the amount of GAPDH mRNA of the P P B cells (the amount of GAPDH mRNA × the frequency of B220 cells and IgA^+ B220 cells obtained by flow cytometric analysis).

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Results

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We examined the immunomodulation of Bacteroides and of Lactobacillus on IgA production by PPs lymphocytes. We determined and compared the total IgA in the supernatants of PPs lymphocytes co-cultured with Bacteroidaceae that B7, B18, and B23, and with lactobacilli that LA, LM and LF for 7 d. Thus, in co-cultured with B7, IgA production was significantly higher than that the medium without bacteria (Medium) (Fig. 1). In addition, mean IgA production in the co-cultures with three strains of Bacteroidaceae (2142.68 ± 284.80 ng/ml, n = 3) was significantly higher than that of IgA^+ B220 cells and IgA^+ B220 cells obtained by flow cytometric analysis. *"p < 0.05.

Fig. 1. The Effects of Bacteroidaceae and Lactobacillus on IgA Production in PPs Lymphocytes.

After pre-culture at 2.5 × 10^5 cells/ml final concentration of PPs lymphocytes for 24 h, 10 μg/ml of lyophilized bacteria were added at the final concentration (day 0), and then co-cultured for 7 d. The supernatant from day 7 was harvested and assayed for total IgA production by ELISA. Data represent the mean ± SD of three individual experiments. *"p < 0.05.

Flow cytometric analysis of cell surface markers on PPs lymphocytes. PPs lymphocytes Fc receptors were blocked with CD16/32 (93; eBioscience, San Diego, CA), and then incubated with optimal concentrations of FITC-conjugated anti-mouse IgA (C10-3; BD Biosciences Franklin Lakes, NJ), PE-conjugated anti-mouse Syn-decan-1 (281-2; BD Biosciences), APC-Cy7-conjugated anti-mouse B220 (RA3-6B2; BD Biosciences), and Propidium Iodide (Sigma Aldrich). Flow cytometric analysis was then performed using FACSCanto (BD Biosciences). Analysis was performed using FlowJo ver.8 (Tree Star, Ashland, OR).
higher than the mean production in those with three strains of lactobacilli (819.99 ± 99.26 ng/ml, n = 3)
(p < 0.01, the statistical remark not shown in Fig. 1).
These results suggest that Bacteroides have stronger ability to induce PPs lymphocyte IgA production than do Lactobacillus.

Bacteroides more strongly induced PPs B cell differentiation to IgA⁺ B cells than Lactobacillus

As shown in Fig. 1, Bacteroides promoted greater IgA production by PPs lymphocytes than Lactobacillus. We hypothesized that this result was due to Bacteroides inducing B cells to differentiate more effectively into IgA-PCs (IgA⁺ B220⁻ Syndecan-1⁺ cells). To test this hypothesis, we examined the change in frequency over time of IgA⁺ B cells and IgA-PCs by flow cytometric analysis of PPs lymphocytes co-cultured with Bacteroidaceae and with lactobacilli for 7 d. The peak number of IgA⁺ B cells was observed at day 5 (Fig. 2A).

Figure 2B shows typical raw data on the frequency of IgA⁺ B cells in PPs lymphocytes co-cultured with Bacteroidaceae and with lactobacilli analyzed by flow cytometry from three independent experiments. In addition, we found the mean from three independent experiments on each bacterium, and then compared Bacteroides and Lactobacillus groups. The data obtained were subjected to statistical analysis (Bacteroides, 2.81 ± 0.45%, n = 3; Lactobacillus, 1.61 ± 0.24%, n = 3; p < 0.05). In addition, increasing numbers of IgA-PCs were observed from day 5. (Fig. 2C).

Figure 2D shows typical raw data on the frequency of IgA⁺ Syndecan-1⁺ cells gated on B220⁻ cells at day 7. Data are representative of three similar individual experiments.
These results indicate that *Bacteroides* differentiation of PPs B cells into IgA+ B cells and subsequent terminal differentiation into IgA-PCs by IL-6 is the cytokine known to induce terminal differentiation from IgA-plasmablasts into IgA-PCs (23,24). Thus the production of these cytokines in supernatants obtained from PPs lymphocytes co-cultured with *Bacteroidaceae* or lactobacilli for 3 d was detected by ELISA. IL-5 production of PPs lymphocytes co-cultured with B23 was significantly higher than with Medium (*p < 0.05*) and LF (*p < 0.05*) (Fig. 4A). In addition, that *Bacteroides* (64.87 ± 33.75 pg/ml, n = 3) tended to induce higher IL-5 production by PPs lymphocytes than *Lactobacillus* (25.90 ± 8.08 pg/ml, n = 3) (*p = 0.124*) (statistical remark not shown in Fig. 4A). In co-culture with B7 (*p < 0.01*) and LF (*p < 0.05*), IL-6 production was significantly higher than Medium (Fig. 4B). However, IL-6 production by PPs lymphocytes was no different between those co-cultured with *Bacteroides* (817.71 ± 151.24 pg/ml, n = 3) and with *Lactobacillus* (700.32 ± 152.51 pg/ml, n = 3) (Fig. 4B). These results suggest that *Bacteroides* did not induce terminal differentiation from IgA-plasmablasts into IgA-PCs by IL-6 with *Lactobacillus* (1.69 ± 0.32, n = 3) (*p < 0.01*, the statistical remark not shown in Fig. 3B). These results suggest that *Bacteroides* induces IgA CSR from μ to α via up-regulation of AID mRNA expression.

**Bacteroides induce more IL-5 production by PPs lymphocytes than Lactobacillus**

IL-5 is the cytokine known to promote μ to α CSR, and IL-6 is the cytokine known to induce terminal differentiation of IgA-plasmablasts into IgA-PCs (23,24). Thus the production of these cytokines in supernatants obtained from PPs lymphocytes co-cultured with *Bacteroidaceae* or lactobacilli for 3 d was detected by ELISA. IL-5 production of PPs lymphocytes co-cultured with B23 was significantly higher than with Medium (*p < 0.05*) and LF (*p < 0.05*) (Fig. 4A). In addition, that *Bacteroides* (64.87 ± 33.75 pg/ml, n = 3) tended to induce higher IL-5 production by PPs lymphocytes than *Lactobacillus* (25.90 ± 8.08 pg/ml, n = 3) (*p = 0.124*) (statistical remark not shown in Fig. 4A). In co-culture with B7 (*p < 0.01*) and LF (*p < 0.05*), IL-6 production was significantly higher than Medium (Fig. 4B). However, IL-6 production by PPs lymphocytes was no different between those co-cultured with *Bacteroides* (817.71 ± 151.24 pg/ml, n = 3) and with *Lactobacillus* (700.32 ± 152.51 pg/ml, n = 3) (Fig. 4B). These results suggest that *Bacteroides* did not induce terminal differentiation from IgA-plasmablasts into IgA-PCs by IL-6 with *Lactobacillus* (1.69 ± 0.32, n = 3) (*p < 0.01*, the statistical remark not shown in Fig. 3B). These results suggest that *Bacteroides* induces IgA CSR from μ to α via up-regulation of AID mRNA expression.

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production, but induced IgA-CSR through IL-5 production as compared with Lactobacillus, and thus IgA-PCs might have been increased (Figs. 2B, 4A and 4B).

Discussion

Previous studies have demonstrated that intestinal commensal bacteria promote IgA production, but it is poorly understood whether the different intestinal commensal bacteria, of which there are 500–1,000 species in the human intestine, have different effects on IgA production. In this study, to determine whether Bacteroides and Lactobacillus, major murine intestinal bacteria, have different effects on IgA production, we compared their IgA inducing abilities in PPs lymphocytes.

AID is identified as the enzyme regulating CSR and somatic hypermutation.① It has been found that IgM B cells but not IgA B cells accumulate in PPs of AID−/− mouse.② In this study, we found that AID mRNA expression in PPs B cells occurred at day 3 when co-cultured with Bacteroidaceae. It was then observed, in PP lymphocytes co-cultured with Bacteroidaceae, that the frequency of IgA B cells at day 5 and of IgA-PCs at day 5 to 7 were increased. On the other hand, in PPs lymphocytes co-cultured with lactobacilli, the expression of AID did not increase and the frequency of IgA B cells was significantly lower than in those co-cultured with Bacteroidaceae. These findings indicate that Bacteroides induce IgA CSR from μ to α in PPs B cells by promoting the expression of AID and IgA production, an effect not seen with Lactobacillus. Furthermore, we observed that the level of IL-5 production by PPs lymphocytes co-cultured with Bacteroidaceae tended to be higher than with lactobacilli. These data suggest that Bacteroides induces IgA production by increasing AID expression via IL-5, in view of the other report that IL-5 increases AID expression in CD38-activated splenic B cells.⑥ IgA-PCs were increased and IgA production might have been elicited.

Some previous reports have indicated that Lactobacillus induced intestinal IgA production,although there was no difference in IgA production between the medium and LA (p = 0.948), LM (p = 0.832), and LF (p = 0.888) (Fig. 1) in our experiments. Almost IgA inducible lactobacilli are derived from humans, but we used murine commensal lactobacilli. This suggests that there might be differences in immunomodulation between lactobacilli derived from murine commensal and other lactobacilli, derived from human microbiota. In this study, we observed that Bacteroides induced higher IgA production by PPs lymphocytes than Lactobacillus. Hence it suggested that there might be inducible bacteria for IgA production in gut commensal bacteria.

It is known that CD11c+ cells migrate to mesenteric lymph nodes (MLN) after phagocytosing luminal antigens, and present them to T and B cells.⑦ Our previous study indicated that Thy1.2− cells isolated from MLN of germ-free mice co-cultured with Bacteroidaceae showed higher expression of MHC II (I-Aδ), CD80 (B7-1), and CD86 (B7-2) than those co-cultured with lactobacilli.⑧ These findings suggest that Bacteroides induces stronger antigen-presentation to T cells than Lactobacillus.

Furthermore, it has been found that oral administration of a fluctuoligosaccharides (FOS) diet to mice for 5 weeks increased the number of Bacteroides. In addition, IgA production increased in PPs lymphocytes obtained from mice administered the FOS diet for 4 weeks.⑨,⑩ These reports indicate that FOS administration increased Bacteroides in the murine intestinal bacteria, with a subsequent increase in IgA production by PPs lymphocytes. Moreover, it is known that LPS promotes B cell differentiation in the presence of transforming growth factor (TGF)-β in vitro.⑪ These findings suggest that in our study, LPS of Bacteroidaceae were also able to modulate the immunoresponses, and that Bacteroides might be the microorganism in the murine intestinal commensal bacteria with the function of promoting mucosal IgA production.

In summary, we found that murine intestinal commensal bacteria induce mucosal IgA production. In particular, Bacteroides, one of the prominent bacteria in the murine commensal bacteria, elicits IgA production, the most important immune response in the gut mucosal immune system. In this study, we found that Bacteroides induce PPs B cell differentiation to IgA+ cells by increasing AID expression, thus inducing IgA production.

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