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Regulation of Intestinal IgA Responses by Dietary Palmitic Acid and Its Metabolism

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Enhancement of intestinal IgA responses is a primary strategy in the development of oral vaccine. Dietary fatty acids are known to regulate host immune responses. In this study, we show that dietary palmitic acid (PA) and its metabolites enhance intestinal IgA responses. Intestinal IgA production was increased in mice maintained on a PA-enriched diet. These mice also showed increased intestinal IgA responses against orally immunized Ag, without any effect on serum Ab responses. We found that PA directly stimulates plasma cells to produce Ab. In addition, mice receiving a PA-enriched diet had increased numbers of IgA-producing plasma cells in the large intestine; this effect was abolished when serine palmitoyltransferase was inhibited. These findings suggest that dietary PA regulates intestinal IgA responses and has the potential to be a diet-derived mucosal adjuvant. The Journal of Immunology, 2014, 193: 1666–1671.

High levels of IgA are present in the intestine, where they protect the host against pathogenic microorganisms by preventing their attachment to and entrance into epithelial cells, as well as by neutralizing their toxins (1). Some patients with IgA deficiency show increased susceptibility to infectious pathogens, including Giardia lamblia, Campylobacter, Clostridium, Salmonella, and rotavirus (2). Given the immunologic importance of IgA in immunosurveillance in the intestine, the primary goal of effective oral vaccines is the efficient induction of Ag-specific IgA responses (3).

An efficient intestinal IgA response requires host-derived factors, including cytokines (e.g., IL-5, IL-6, IL-10, IL-15, APRIL, BAFF) and chemokines (e.g., CCL25/CCR9) (4, 5), as well as immunologic cross-talk with environmental factors (e.g., commensal bacteria and dietary materials) (6). Indeed, germ-free mice have decreased intestinal IgA responses because of the immature structure of Peyer’s patches (PPs) and isolated lymphoid follicles (7, 8). We recently identified a unique subset of intestinal IgA-producing plasma cells (PCs) in the murine intestine; these cells expressed CD11b, required microbial stimulation and mature PP structure, proliferated vigorously, and produced high amounts of IgA (9).

In addition to commensal bacteria, nutritional molecules, such as vitamins, are essential for the development, maintenance, and regulation of intestinal immune responses (6, 10, 11). Therefore, nutritional deficiencies and inappropriate dietary intake increase the risk for infectious, allergic, and inflammatory diseases (12, 13). Among various dietary factors, oils are known to influence host immune function and inflammatory responses (14–16). Overnutrition due to a high-fat diet leads to the development of inflammation in adipose tissue, which is frequently associated with obesity and atherosclerosis (17). Recent findings suggest that, in addition to the quantity of oil ingested, the fatty acid (FA) composition of the oil is an important factor in various immunologic and inflammatory conditions (14–16). Dietary oil is generally composed of long-chain saturated FAs (e.g., C16:0 palmitic acid [PA] and C18:0 stearic acid) and mono- or polyunsaturated FAs (PUFAs; e.g., C18:1 oleic acid, C18:2 linoleic acid, and C18:3 ω-3-linolenic acid). ω-3 Linolenic acid and linoleic acid are precursors of ω3 and ω6 PUFAs, respectively; ω3 FAs are metabolized into anti-inflammatory molecules, whereas ω6 FAs are converted into proinflammatory lipid mediators (16, 18). Therefore, the ratio of ω-3-linolenic acid/linoleic acid in dietary oils is thought to determine the onset of various immunologic conditions. In addition to modulating the ω3–ω6 PUF balance, saturated FAs, such as PA, stimulate host immune responses by promoting the production of proinflammatory cytokines, including IL-6 and TNF-α (19, 20). Although these proinflammatory cytokines are prerequisite factors for the efficient induction of IgA...
responses (5), the immunologic function of dietary PA in the control of IgA production remained to be investigated. In this study, we show that PA-enriched diets enhance intestinal IgA responses both directly and through their metabolic pathways.

Materials and Methods

Mice
Female BALB/c, C3H/HeJ, and C3H/HeN mice were purchased from CLEA Japan (Tokyo, Japan). Chemically defined AIN-93M–based diets containing soybean, palm, or coconut oil or soybean oil plus supplemental purified PA were from Oriental Yeast (Tokyo, Japan). All mice were provided with a sterile diet and water ad libitum. To inhibit serine palmitoyltransferase (SPT) activity, mice were treated with myriocin (1.0 mg/kg i.p. daily; Sigma-Aldrich, St. Louis, MO) for 4 d (21). All mice were maintained in the experimental animal facility at the University of Tokyo and National Institute of Biomedical Innovation; the experiments were approved by the Animal Care and Use Committee of each institute and conducted in accordance with their guidelines.

Oral immunization
Mice received sodium bicarbonate to neutralize gastric acid before oral immunization (9, 22). Thirty minutes later, the mice were immunized orally concurrently with 1 mg OVA (Sigma-Aldrich) and 10 μg cholera toxin (CT; List Biological Laboratories, Campbell, CA). This oral-immunization procedure was conducted on days 0, 7, and 14.

Cell isolation
To isolate mononuclear cells from PPs, we stirred intestinal tissues in RPMI 1640 medium containing 2% FCS and 0.5 mg/ml collagenase (Wako, Osaka, Japan). Cells were isolated from the intestinal lamina propria (iLP) as previously described (9, 22). Briefly, PPs were removed, and small and large intestines were cut into 2-cm pieces, which were stirred in RPMI 1640 containing 1 mM EDTA and 2% FCS. Then the tissues were stirred in collagenase for 15 min three times (small intestine, 0.8 mg/ml; large intestine, 1.6 mg/ml) before undergoing discontinuous Percoll gradient centrifugation. Lymphocytes were isolated at the interface between the 40 and 75% layers.

Detection of total Ig by ELISA and OVA-specific Ab responses by ELISPOT assay
Total Ig levels in serum and fecal extracts were determined by ELISA, as previously described (9, 22). To measure Ab concentration, purified murine isotype-specific Abs (BD Biosciences, San Jose, CA) were used as standards for quantification. For the detection of OVA-specific Abs and Ab-forming cells, fecal extracts and iLP were prepared 7 d after the final immunization. Standard OVA-specific ELISAs and ELISPOT assays were performed as previously described (9, 22).

Flow cytometry and cell sorting
Flow cytometry and cell sorting were performed as previously described (9, 22). Cells were preincubated with anti-CD16/32 Ab and then stained with fluorescent Abs specific for B220, IgG1, IgA (all from BD Biosciences), CD19, and CD138 (BioLegend, San Diego, CA). Forward scatter–height and forward scatter–area discrimination was used to exclude doublet cells, and Via-Probe Cell Viability Solution (BD Biosciences) was used to discriminate between dead and living cells. For the BrdU-uptake assay, mice received 1 mg BrdU i.p., and the BrdU signal was detected according to the manufacturer’s protocol (BD Biosciences) (9). Concentration-matched isotype Abs were used as negative controls. Flow cytometric analysis and cell sorting were carried out using FACSCanto II and FACS aria (BD Biosciences, respectively). We confirmed that cell purity was ~95%.

Results

Mice maintained on a diet containing palm oil show enhanced intestinal IgA production
To examine whether dietary PA affects intestinal IgA production, we maintained mice on a diet containing 4% soybean (control) or palm (PA-rich) oil for 2 mo (Fig. 1A) and measured the amounts of fecal IgA. Intestinal IgA production was higher in the mice that received palm oil than in those given soybean oil (Fig. 1B). In contrast, the amounts of serum IgG and IgA were similar between the soybean and palm oil groups (Fig. 1B, Supplemental Fig. 1). Palm oil is high in both PA and oleic acid (Fig. 1A, 1B). Both groups had similar levels of total Ig in serum, and no differences were observed in the splenic B cell populations (data not shown).

We then considered whether other saturated FAs enhance intestinal IgA production. To this end, we used coconut oil, which (like palm oil) is a Palmae plant–based oil but contains large amounts of saturated FAs. Coconut oil is high in both PA and myristic acid (Fig. 1A). Intestinal IgA production was not significantly different between mice maintained on a diet containing coconut oil and those maintained on a diet containing palm oil (Fig. 1B). In contrast, serum IgG levels were higher in the coconut oil group than in the palm oil group (Fig. 1B). These results suggest that the presence of oleic acid enhances intestinal IgA production, whereas the presence of PA does not.

FIGURE 1. Palm oil enhances intestinal IgA production. (A) FA composition of soybean, palm, and coconut oils. (B) Mice were maintained on a diet containing soybean, palm, or coconut oil for 2 mo. Feces and serum samples were collected for measurement of the amounts of IgA and IgG, respectively (mean ± 1 SD, n = 10/group).

Statistics
Experimental groups were compared using the Mann–Whitney U test (GraphPad, San Diego, CA).

Materials and Methods
amounts of other saturated FAs, including lauric and myristic acids (Fig. 1A). Unlike palm oil, coconut oil did not alter fecal or serum Ab production in the mice (Fig. 1B). These findings suggest that dietary PA uniquely enhances intestinal IgA production.

**PA-enriched dietary oils enhance intestinal IgA production and IgA responses against oral vaccine Ag**

To confirm that PA enhances intestinal IgA production, we added PA to soybean oil to adjust its PA concentration to that of palm oil (Fig. 2A, upper bar). Fecal IgA levels in mice maintained for 2 mo on a diet containing the PA-enriched soybean oil were increased similarly to those of mice fed a palm oil–containing diet, but serum IgA production was unchanged (Fig. 2A, lower bar, Supplemental Fig. 1). These data suggest that PA is a key FA in the enhancement of intestinal IgA production.

We then investigated whether the enhanced production of intestinal IgA induced by dietary PA is reflected in the responses to an orally administered vaccine. To this end, we orally immunized mice concurrently with OVA and the mucosal adjuvant CT. In agreement with levels of naturally produced IgA, OVA-specific fecal IgA responses were enhanced in mice maintained on a diet that contained palm oil (Fig. 3). In addition, similarly increased OVA-specific IgA production was noted in mice maintained on PA-enriched soybean oil (Fig. 3). These findings suggest that dietary PA enhanced not only naturally produced intestinal IgA but also Ag-specific intestinal IgA induced by oral immunization.

**PA content is increased in the intestinal, but not systemic, compartment**

We next measured the amounts of PA in the intestines and serum of the mice. PA concentrations in the small and large intestines were higher in the mice maintained on PA-rich soybean oil compared with soybean oil alone (Fig. 4). In contrast, PA amounts in serum were comparable between groups (Fig. 4). These findings indicate that dietary PA affects the amount of PA locally in the intestine without any influence on serum PA concentration.

**PA directly stimulates intestinal PCs to produce IgA**

We then examined whether PA directly affected IgA production from PCs. To address this issue, we purified IgA+ PCs from the intestine and cultured them with PA for 4 d. The amount of IgA in the intestinal PC culture supernatants increased in a dose-dependent manner (Fig. 5A). ELISPOT assays showed that PA did not increase the number of IgA-forming cells (Fig. 5B), suggesting that PA instead enhances Ab production from PCs. In addition, IgG production from CD19+ CD138+ PCs from the spleen was increased in the presence of PA (Supplemental Fig. 2), indicating that PA enhances Ab production, regardless of the Ig subtype.

PA acts as a ligand for TLR4 (24), prompting our investigation into whether TLR4 mediated the PA-induced direct activation of IgA PCs. To address this issue, we used C3H/HeN and C3H/HeJ mice. Although TLR4-mediated signaling differs between these two strains because of a spontaneous point mutation in the Tlr4 gene of the C3H/HeJ mice (25, 26), intestinal PCs from these mouse strains produced identical levels of PA-induced IgA (Fig. 5C). Therefore, the direct effect of PA on IgA production from PCs likely is independent of the TLR4 pathway.

**The PA-induced increase in IgA PCs in the large intestine is dependent on SPT**

We used flow cytometry to determine the frequency of IgA+ PCs in the small and large intestines. Although no significant difference between the control and PA-enriched diets was noted in the small intestine, the frequency of IgA+ B220+ PCs was increased in the

**FIGURE 2.** Dietary PA alone is sufficient to enhance intestinal IgA production. (A) Purified PA was added to soybean oil to achieve the same PA content as that in palm oil. (B) Mice were maintained for 2 mo on a diet containing soybean oil, with or without supplemental PA. The IgA levels of fecal extracts were measured (mean ± 1 SD, n = 16/group).

**FIGURE 3.** Ingestion of PA-enriched oil enhances intestinal IgA responses against orally immunized Ag. After mice were maintained for 2 mo on a diet containing soybean oil, soybean oil + PA, or palm oil, they were orally immunized with OVA plus CT on days 0, 7, and 14. On day 21, fecal extracts were collected for the determination of OVA-specific intestinal IgA by ELISA. Data are mean ± 1 SD (n = 4/group), and similar results were obtained from two independent experiments. *p < 0.05.

**FIGURE 4.** Increased PA content in the intestine of mice maintained on PA-enriched oils. Mice were maintained for 2 mo on a diet containing soybean oil, with or without PA, and small and large intestinal tissues and serum were collected for measurement of their PA contents. The graphs show data from individual mice, and the horizontal lines indicate the means; similar results were obtained from two independent experiments.
large intestine of the PA-enriched diet group (Fig. 6A). Similarly, the number of OVA-specific IgA-forming cells was increased in the large, but not small, intestine of mice receiving oral immunization and the PA-enriched diet (Fig. 6B). In agreement with the lack of a PA-associated effect on small intestinal IgA, B cell differentiation into IgA+ cells in the PPs, a lymphoid tissue for the initiation of small intestinal IgA responses (3), was unchanged in mice maintained on a PA-enriched diet (Supplemental Fig. 3).

PA can be converted into sphingolipids, including ceramide, sphingosine, and sphingosine 1 phosphate (S1P) (Fig. 6C); all of these lipids all known to promote cell proliferation, survival, and trafficking (27, 28). Therefore, we supposed that these PA-derived sphingolipids might be involved in the regulation of intestinal IgA PCs in the large intestine. To test this possibility, mice were treated with myriocin, an inhibitor of SPT, which is a key enzyme in the conversion of PA into sphingolipids (Fig. 6C). The PA-mediated increase in IgA PCs did not occur in the large intestine of mice that received myriocin (Fig. 6D), and myriocin had little effect on the number of IgG1+ cells in the spleen (Supplemental Fig. 4), suggesting that SPT activity is required for this effect on large intestinal IgA PCs.

FIGURE 5. PA directly stimulates IgA-producing PCs via a TLR4-independent mechanism. IgA+ B220<sup>−</sup> PCs were isolated from the small intestine of BALB/c (A and B) and C3H/HeN or C3H/HeJ mice (C) and cultured with 10 or 100 μM (A) or 100 μM (B and C) PA. The amount of IgA in the culture supernatant (A and C) and the number of IgA+ Ab–forming cells (IgA+-AFCs) (B) were determined by ELISA and ELISPOT, respectively. The graphs show data from individual samples, and the horizontal lines indicate the means; similar results were obtained from two independent experiments (A and C). Data in (B) are mean ± 1 SD (n = 10/group from two separate experiments).

FIGURE 6. SPT is required for the increased number of IgA PCs in the large intestine. Mice were maintained on a diet containing soybean oil, with or without PA, for 2 mo. (A) Mononuclear cells were isolated from the small and large intestines for the flow cytometric analysis of IgA+ B220<sup>−</sup> PCs. Data are representative of three independent experiments. (B) Mice were orally immunized with OVA plus CT on days 0, 7, and 14. On day 21, mononuclear cells were isolated from the small and large intestine for the detection of OVA-specific IgA Ab–forming cells by ELISPOT assay. Data are mean ± 1 SD (n = 6/group from two separate experiments). (C) The metabolic pathway mediated by SPT to generate sphingolipids from PA. (D) The frequency of IgA+ B220<sup>−</sup> PCs in the large intestine of mice maintained on a PA-enriched diet and myriocin. The graphs show data from individual samples, and the horizontal lines indicate the means. Similar results were obtained from two independent experiments.
The increased proliferation of IgA PCs in the large intestine of mice maintained with a PA-enriched diet

We performed BrdU-uptake assays to examine the effects of PA on the proliferation and survival of IgA PCs in the large intestine. Soon after BrdU administration (day 1), the proportions of BrdU+ and BrdU− IgA+ cells in the large intestine were higher when mice were maintained on a PA-enriched diet than when they were maintained on the control diet, but the increase ratio of IgA+ cells was higher in BrdU+ IgA+ cells than in BrdU− IgA+ cells (Fig. 7A). These findings suggest that PA metabolites induced the proliferation of IgA+ cells in the large intestine. In contrast, on day 4, both the control and PA-enriched groups showed similar levels of BrdU+ IgA+ cells (Fig. 7B). These data collectively suggested that PA metabolites primarily induced the proliferation of IgA+ cells in the large intestine rather than prolonged their survival.

Discussion

In the current study, we extended our knowledge of lipid-mediated immune regulation by showing the immunologic function of dietary PA in the enhancement of intestinal IgA responses. Unlike the sterile environment of systemic immune compartments (e.g., spleen), intestinal tissues are continuously exposed to exogenous factors, including commensal bacteria and dietary materials and actively use them to establish a homeostatic inflammatory condition (6). Indeed, in contrast to the massive inflammatory responses induced by the systemic injection of bacterial products, such as LPS (e.g., sepsis), the intestinal immune system requires bacterial stimulation for its maturation (29). Our current study demonstrates that dietary PA can augment intestinal IgA responses when an appropriate amount of dietary oil (4%) is supplied. This effect contrasts sharply with the deleterious plasma PA levels that are induced by a high-fat diet and which are considered to be a risk factor for inflammation and diabetes (30). Therefore, like bacterial products, PA has the opposite immunologic effect on intestinal and systemic immune compartments and actually plays a beneficial role in the maturation of the intestinal immune system.

The enhancement of intestinal IgA responses by dietary PA is mediated by at least two distinct pathways. One is PA’s direct effect on IgA-producing PCs, and the other is mediated by PA-derived metabolites, sphingolipids. In addition to these two pathways, PA affects APCs (e.g., macrophages and dendritic cells) to promote Ag presentation and the production of cytokines, including IL-6 and TNF-α; this effect is at least partly mediated by TLR4 (31, 32) and represents a plausible third pathway to enhancing intestinal IgA production. Unlike the effect of PA on APCs, PA promoted IgA production from PCs in a TLR4-independent manner in our current study. In line with this finding, several groups reported that TLR2 acts as a receptor for PA or that PA enters cells where it induces signal transduction for the consequent production of inflammatory cytokines (33–36).

In addition to PA’s direct effect on IgA-producing PCs, the SPT-mediated metabolism of PA is involved in the PA-mediated enhancement of intestinal IgA responses. SPT is an essential enzyme for the de novo pathway of sphingolipid synthesis, in which palmitoyl-CoA and serine act as substrates of SPT to generate 3-keto-dihydrosphingosine, with subsequent conversion into other sphingolipids, such as sphingomyelin, ceramide, sphingosine, and S1P (37, 38). Sphingolipids are a class of membrane lipids that also are known to have biologic functions (27, 28). For example, ceramide regulates cytoskeletal changes, cell cycle, and apoptosis (27, 28). Accordingly, perhaps an increase in ceramide concentrations induced the proliferation or prolonged the survival of IgA PCs, subsequently increasing the number of IgA-producing PCs in the large intestine. In addition, in the extracellular compartment, S1P controls cell trafficking by recruiting cells toward regions with high concentrations of S1P (39). Of note, we previously reported that S1P regulates intestinal IgA responses by controlling trafficking of IgA+ cells from inductive sites (e.g., PP and peritoneal cavity) into the iLP (22, 40). Therefore, another possibility is that the PA-enriched diet induced an increase in the intestinal extracellular S1P concentration, resulting in the effective recruitment of IgA PCs into the intestine. Our current findings suggest that at least one of the enhancing effects of the PA-enriched diet was mediated by the induction of proliferating IgA+ PCs in the large intestine.

PA was not only included in the diet but was also generated through de novo lipogenesis, whereby carbohydrates are converted to PA. The de novo pathway achieves stable concentrations of PA in vivo. This pathway can explain the specificity of the effect of dietary PA enrichment on the PA content in various tissues. In this study, we found that the increases in PA in the PA-enriched diet group were specific to the intestinal tissues and not the serum. This tissue specificity is consistent with the specific effect of dietary PA, which increased intestinal IgA responses without affecting serum Ab production. Mice fed high-fat diets show increased serum PA levels, which are a risk factor for inflammation and diabetes (30). However, our current findings indicate that the increased proportion of PA in the dietary oil did not affect serum PA levels when the overall amount of oil consumed was normal (4%).

Intestinal tissues contain higher levels of sphingolipids than do other tissues (41), and we found that the effect of an SPT inhibitor was selective for the large, but not small, intestine. One of the major differences between the small and large intestines is the amount of commensal bacteria. We recently reported that several lipid metabolic pathways are uniquely mediated by commensal bacteria (42), raising the possibility that commensal bacteria may affect sphingolipid metabolism. However, germ-free and specific pathogen-free rats have comparable levels of sphingolipids in the intestine (43). Therefore, it is plausible that commensal bacteria are unlikely to participate in this pathway. In contrast, the expression pattern of enzymes involved in the generation of sphingolipids differs between intestinal compartments (44, 45), indicating that the differences in sphingolipid metabolism between the small and large intestines determine their differing dependence on SPT in PA-mediated intestinal IgA responses. Taken together, our current findings demonstrate that dietary PA and its metabolites play a critical role in the enhancement of intestinal IgA responses. This information can be applied to the development of mucosal adjuvants.

FIGURE 7. A PA-enriched diet induces the proliferation of IgA PCs. Mice were maintained on a diet containing soybean oil, with or without PA, for 2 mo, after which BrdU was injected i.p. (day 0). On day 1 (A) and day 4 (B), mononuclear cells isolated from the large intestine were analyzed by flow cytometry to determine the proportion of BrdU+ IgA+ PCs. Data are mean ± 1 SD (n = 4). Similar results were obtained from two independent experiments.