Dietary Plasma Proteins Modulate the Immune Response of Diffuse Gut-Associated Lymphoid Tissue in Rats Challenged with Staphylococcus aureus Enterotoxin B1,2

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Abstract
We have previously shown that plasma protein supplementation prevents the activation of lymphocyte populations of Peyer’s patches and mesenteric lymph nodes, which is known as organized gut-associated lymphoid tissue (GALT). Here, we examined the effects of spray-dried plasma proteins (SDAP) and Ig concentrate (IgC) supplements on lamina propria and intraepithelial lymphocytes (diffuse GALT) in a model of mild intestinal inflammation induced by the intraperitoneal administration of Staphylococcus aureus enterotoxin B (SEB). Wistar-Lewis rats were fed diets supplemented with SDAP (8% wt:wt), IgC (1.5% wt:wt), or bovine milk proteins (control diet) from weaning (d 21) to d 34 after birth. On d 30 and 33, rats were given SEB (0.5 mg/kg body weight) or PBS (control). Experimental groups were designated control, SEB, SEB-SDAP, and SEB-IgC. Lymphocyte populations were analyzed by immunohistochemistry. In lamina propria, SEB increased the cytotoxic lymphocyte populations of T-gd cells (38%; P < 0.001) and natural killer cells (59%; P < 0.05) and the number of activated T lymphocytes (148%; P < 0.001). Both SDAP and IgC decreased the effects of SEB on these lymphocyte subsets (P < 0.05). In the epithelium, SEB induced a 117% increase in intraepithelial-activated lymphocytes that was reduced by SDAP supplementation (P < 0.01). The effects of plasma supplements on intestinal lymphocyte populations suggest that oral plasma proteins can modulate the degree of activation of diffuse GALT. J. Nutr. 138: 533–537, 2008.

Introduction
The mucosal immune system interacts with host and intestinal microbiota and plays an important role in protecting against pathogenic microorganisms. The mucosal immune system maintains homeostasis by innate and acquired immunity along the epithelial surface (1). Gut-associated lymphoid tissue (GALT)6 accounts for up to 80% of the mucosal immune system and is distributed throughout the intestine either as organized GALT, in the form of Peyer’s patches, isolated follicles, and mesenteric lymph nodes, or as diffuse GALT, which consists of lymphocytes scattered along the epithelium and the lamina propria (2). Both compartments are part of a regulatory pathway with specific differentiated roles: organized GALT is the inductor site of the immune response and diffuse GALT is the effector site. Staphylococcal exotoxins are potent activators of the immune system that can stimulate a high percentage of T cells by cross-linking major histocompatibility complex class II molecules with specific regions of the β-chain of the T-cell receptor (3), hence the name “superantigens.” They cause a variety of diseases in animals and humans, ranging from food poisoning to shock. The intraperitoneal administration of low doses of Staphylococcus aureus enterotoxin B (SEB) to rats at d 30 and 33 after birth induces a mild inflammatory response characterized by increased water secretion, neutrophil infiltration in intestinal mucosa, and increased intestinal permeability (4,5). SEB also increases the number of T lymphocytes, the percentage of activated T lymphocytes that was reduced by SDAP supplementation (P < 0.01). The effects of plasma supplements on intestinal lymphocyte populations suggest that oral plasma proteins can modulate the degree of activation of diffuse GALT.

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6 Abbreviations used: GALT, gut-associated lymphoid tissue; IEL, intraepithelial lymphocytes; IgC, Ig concentrate; IL-2R, interleukin-2 receptor; NK cell, natural killer cell; SDAP, spray-dried plasma proteins; SEB, Staphylococcus aureus enterotoxin B.
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improves animal growth and performance in farm animals challenged with pathogenic *Escherichia coli* (8) and animals in unsanitary environments (9). In rats challenged with SEB, both spray-dried plasma proteins (SDAP) and Ig concentrates (IgC) prevent the proliferation of activated T helper lymphocytes in Peyer’s patches and mesenteric lymph nodes (4). Plasma protein supplementation reduces the number of intraepithelial lymphocytes (IEL) and cell density in the lamina propria of the large intestine of pigs (10). It has been demonstrated that plasma supplements modulate the immune response of organized GALT (4). Therefore, the aims of this study were to characterize the pathophysiological features of the SEB model at the lamina propria and intraepithelial compartments of the GALT and to evaluate the effects of diets supplemented with SDAP and IgC on lymphocyte cell populations of diffuse GALT in the SEB model of intestinal inflammation.

**Methods**

**Animals and diets.** Male Wistar Lewis rats supplied by Harlan Ibérica were kept under stable temperature and humidity conditions, with a 12-h light/dark cycle and free access to food and water. All protocols used in this study were approved by the ethical committees for animal experimentation of the University of Barcelona and the Regional Government.

At d 21 after birth, rats were weaned and distributed to the different experimental groups (n = 5–8 rats per group). They were then fed the experimental diets until d 34. Groups included: 1) Control: rats fed the control diet and given PBS; 2) SEB: rats fed the control diet and treated with SEB; 3) SEB-SDAP: rats fed a diet containing 80 g/kg SDAP and treated with SEB; and 4) SEB-IgC: rats fed a diet containing 15 g/kg porcine IgC and treated with SEB. The effective daily intake of dietary supplements between d 21 (weaning) and the end of the experiment (d 33–35) ranged from 480 to 800 mg/d for SDAP and from 90 to 150 mg/d for IgC. Both SDAP and IgC were derived from the same batch of fresh porcine IgC and treated with SEB. The pellets were spray-dried to obtain a stable powder product containing active Ig. Maintenance of the native structure was checked using immunoelectrophoresis and Western blotting.

Pelleted diets were formulated to meet the NRC requirements (11) for laboratory animals (Table 1). They were isoenergetic, isonitrogenous, and had the same lysine content. SDAP is a feed ingredient obtained by centrifuging the RBC of hygienically collected porcine blood from pigs (12). IgC was obtained by purifying plasma Ig (13). Both ingredients were spray-dried to obtain a stable powder containing active Ig. Maintenance of the native structure was checked using immunoelectrophoresis and Western blotting. Activity was verified by an ELISA that specifically recognizes *E. coli* (14). Diets supplemented with SDAP or IgC were formulated to have a similar IgG content (10 g/kg diet).

SEB (Toxin Technology) was dissolved in PBS and administered intraperitoneally to rats at d 30 and 33 after birth at a dose of 0.5 mg/kg body weight, as previously described (5).

**Immunohistochemistry of different lymphocyte populations.** Rats were killed at d 34. The jejunal was then excised, flushed with PBS, and cut into 0.5-cm fragments. Fragments were embedded in a drop of Tissue-Tek O.C.T. Compound (Miles), placed on a piece of fine cardboard, and immediately submerged into isopentane. They were then stored at −80°C. Blocks were cut with a cryostat CM3050S (Leica Microsystems). Jejunal tissue sections were permeabilized with a solution containing 10 g/L bovine serum albumin (Sigma) and Triton X-100 0.1% (v:v; Sigma) in PBS at room temperature for 30 min. The slices were incubated overnight at 4°C with a solution containing 10 g/L bovine serum albumin and the corresponding primary mouse monoclonal antibody in a humidified chamber. The primary antibodies used were anti-CD3 (G4.18, Pharmingen), anti-CD4 (W3/25, Labgen), anti-CD8 (OX8, Labgen), anti-γδ T cell receptor (V65, Pharmingen), antireceptor for interleukin 2 (IL-2R, also called CD25, NDS61, Labgen), and anti-NKR-P1A (10/78, Pharmingen). Sections were washed with PBS and incubated with a biotinylated secondary antibody. They were then washed with PBS and incubated with horseradish peroxidase conjugated streptavidin (Histomouse-Plus kit, Zyomed) in a humidified chamber for 30 min at room temperature. They were rinsed in PBS and incubated with tyramide amplification signal (Perkin Elmer), a peroxidase substrate that develops fluorescence instead of visible color. Afterwards, the samples were washed in PBS and counterstained with nuclear marker Hoechst 33342 (Calbiochem) for 5 min at room temperature. They were then washed again with PBS and mounted in Mowiol-488 (Calbiochem). Negative controls were performed without the primary antibodies. The samples were stored at 4°C until observation by confocal microscopy.

**Confocal scanning laser microscope and image processing.** Digital fluorescence images were acquired by confocal scanning laser microscope SPII (Leica Microsystems). Five different fields for each cellular staining were analyzed in a blinded fashion, resulting in a minimum of 30 images per animal. The captured images were analyzed using the NIH image program (15) to measure the villous area and perimeter and to quantify the cell number in each area unit. Results of lamina propria lymphocytes are expressed as cell number per surface area (in millimeters²) and IEL are expressed as cell number per linear millimeter.

**Statistical analyses.** Results are given as means ± SEM of 5–8 rats. To analyze the effect of the enterotoxin administration, the SEB rats were compared with the control rats by an ANOVA using SPSS-11.0 software. To study the effect of dietary supplements on intestinal inflammation, the rats fed the supplemented diets (SEB-SDAP, SEB-IgC) were compared with the rats fed the nonsupplemented diet (SEB) by another ANOVA, followed by a Scheffé post hoc test. Differences were considered significant at *P* < 0.05.

**Results**

Jejunums from rats of the 4 conditions appeared histologically normal and no mucosal damage was observed (Fig. 1).

**T lymphocytes and activated T lymphocytes.** In the lamina propria compartment, SEB treatment increased the number of T lymphocytes (*P* < 0.001; Fig. 2A). Both the SDAP and the IgC-supplemented diets reduced the effects of SEB in this compartment (both *P* < 0.01). The SEB group also had more T lymphocytes in the intraepithelial compartment than the control group (Table 2). Supplemented diets did not affect the numbers of intraepithelial T lymphocytes induced by SEB administration.

Activated T lymphocytes express the IL-2R in their surface. The number of activated lymphocytes in the lamina propria compartment of SEB rats was 148% higher than in the control

<table>
<thead>
<tr>
<th>Table 1 Composition of experimental diets</th>
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<tbody>
<tr>
<td><strong>Ingredients</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>SDAP</td>
</tr>
<tr>
<td>IgC</td>
</tr>
<tr>
<td>Corn starch</td>
</tr>
<tr>
<td>Dried skim milk</td>
</tr>
<tr>
<td>Sugar</td>
</tr>
<tr>
<td>Soybean oil</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>AIN-93 VM³</td>
</tr>
<tr>
<td>AIN-93 MX³</td>
</tr>
<tr>
<td>DL-Methionine</td>
</tr>
<tr>
<td>Choline bitartrate</td>
</tr>
</tbody>
</table>

1 SDAP, AP-820. APC-Europe.
2 APC-Europe.
3 Provided by Harlan Ibérica.

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*E. coli* improves animal growth and performance in farm animals...
Both supplemented diets attenuated the SEB-induced proliferative effects on lamina propria-activated lymphocytes (P < 0.05). SEB treatment also induced a 117% increase in intraepithelial-activated lymphocytes (CD25<sup>+</sup>) compared with control rats (Table 2). The SDAP-supplemented diet decreased the effects of SEB on the number of intraepithelial activated lymphocyte subset, whereas SEB-IgC rats did not differ from the SEB group.

**T helper lymphocytes and T suppressor/cytotoxic lymphocytes.** T lymphocytes can be divided into 2 main subpopulations: CD4<sup>+</sup> lymphocytes (also called T helper lymphocytes) and CD8<sup>+</sup> lymphocytes (T suppressor/cytotoxic lymphocytes).

There were more T helper lymphocytes in SEB rats than in control rats (P < 0.001; Fig. 2A). Both supplemented diets diminished a SEB-induced increase in the lamina propria T helper subset (both P < 0.05). In the intraepithelial compartment, T helper lymphocytes represent a very small cell subset. Neither SEB administration nor diet supplementation modified the population of intraepithelial T helper lymphocytes (Table 2).

Quantification of lamina propria CD8<sup>+</sup> T lymphocytes from the control group indicated that there were 200 ± 21 cells/mm<sup>2</sup> (Fig. 2A). Treatment with SEB increased the number of CD8<sup>+</sup> lymphocytes in this compartment (P < 0.01). Values in the dietary supplement groups were lower than those obtained in the SEB group (P < 0.05). The number of intraepithelial T suppressor/cytotoxic lymphocytes was increased by SEB (P < 0.05). However, neither the SDAP nor the IgC dietary supplement modified the SEB effects on this lymphocyte subset (Table 2).

**Natural killer cells and T<sub>γδ</sub> lymphocytes.** Natural killer cells (NK cells) belong to the innate immune defense mechanism and develop mainly cytotoxic functions. T<sub>γδ</sub> lymphocytes belong to the acquired immune mechanism and also have a cytotoxic function.
function. Together with the NK cells, Tγδ lymphocytes are important in the intraepithelial compartment.

SEB administration increased the number of NK cells in the lamina propria compartment by 59% (Fig. 2B). This increase was reduced by both dietary supplements, although the effects of SDAP (P < 0.01) were greater than the effects of the IgC supplement (P < 0.05). The effects of the 2 plasma supplements differed in this variable (SEB-SDAP vs. SEB-IgC, P = 0.01). In the intraepithelial compartment, only rats treated with SEB experienced changes in the number of NK cells; no dietary effect was observed (Table 2).

SEB treatment induced a 38% increase in lamina propria Tγδ lymphocytes (P < 0.001; Fig. 2A). Both dietary supplements decreased the SEB effects on Tγδ lymphocytes present in the lamina propria (P < 0.01). SEB treatment increased the basal Tγδ lymphocyte number by 68% in the intraepithelial compartment (P < 0.001). Neither SDAP- nor IgC-supplemented diets modified SEB-induced effects on the intraepithelial Tγδ lymphocyte subset (Table 2).

**Discussion**

SEB induced significant mobilization of lymphocytes and NK cells in the lamina propria and in the epithelium, reflecting an inflammatory process that can be prevented by SDAP and, to a lesser extent, IgC supplementation. This effect was consistently observed in GALT populations of the lamina propria, indicating that this mucosal compartment is a target of the regulatory effects of plasma proteins.

Bacterial superantigens are potent proinflammatory stimuli. They can alter gut function as they evoke an acute inflammatory response that may become chronic (3). SEB and related superantigens strongly activate the cellular immune response by stimulating most of the T cells bearing particular T cell receptor Vβ domains while bypassing the restricted presentation of conventional antigens in the major histocompatibility complex (16,17). In this study, SEB induced T lymphocyte activation in the lamina propria, one of the constituents of GALT that participates in the effector phase of the intestinal immune response. In this mucosal compartment, SEB increased the number of T cells [mainly activated T cells (CD25+)], reflecting an overstimulation of the immune system. In addition, other immune population subsets, such as T suppressor/cytotoxic lymphocytes or T helper lymphocytes, also increased. T helper lymphocytes are the main factor responsible for SEB-induced intestinal immune activation (18).

The SEB-induced increase in the number of T suppressor/cytotoxic lymphocytes might reflect an increased number of cells with cytotoxic activity. Separate analyses of small intestine cell populations with this activity indicated that certain cytotoxic populations, such as NK cells and Tγδ-lymphocytes, can also contribute to the response. NK cells are susceptible to direct SEB stimulation, because they can express the MHC class II molecule and therefore can act as antigen-presenting cells (19). Although cytokine production was not measured in this set of experiments, it is well known that SEB induces a rapid increase in circulating cytokines (IL-2, IL-6, interferon-γ), whose value returns to normal 24 h after the antigen challenge (20).

In a previous study (4), diets supplemented with SDAP or IgC did not prevent the SEB-induced increase in mucosal myeloperoxidase activity. However, the general trend in our results was that both dietary supplements reduced the SEB activation of lymphoid tissue associated with the lamina propria. These results are consistent with the observation (4) that dietary supplementation with plasma proteins can reduce the immune activation of Peyer’s patches and mesenteric lymph nodes (the inductor GALT). Both dietary plasma supplements also reduced the pathophysiological responses due to GALT activation by SEB. This response includes a reduction in nutrient transport (21) and increased mucosal permeability (5). Our results are consistent with the view that superantigens are potent mitogens for lamina propria lymphocytes (22). However, the effects of SEB on IEL were modest, probably because the T helper lymphocytes are a minority subset in this compartment (23). The main intraepithelial populations are Tγδ lymphocytes, expressing the conventional receptor CD8, and lymphocytes that express CD8αα homodimer (23). The effect of dietary supplements on lymphocyte

**TABLE 2** Quantification of IEL in the jejunum of control rats and rats challenged with SEB and fed diets supplemented with SDAP and IgC

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>SEB</th>
<th>SEB-SDAP</th>
<th>SEB-IgC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>5</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>CD3+</td>
<td>7.3 ± 0.9</td>
<td>9.6 ± 0.5*</td>
<td>10.6 ± 0.5</td>
<td>9.2 ± 1.2</td>
</tr>
<tr>
<td>CD4+</td>
<td>1.6 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.8 ± 0.3</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>CD8+</td>
<td>5.8 ± 0.8</td>
<td>7.9 ± 0.3*</td>
<td>8.8 ± 0.5</td>
<td>6.8 ± 1.0</td>
</tr>
<tr>
<td>CD25+</td>
<td>0.6 ± 0.1</td>
<td>1.3 ± 0.2*</td>
<td>0.8 ± 0.1b</td>
<td>1.4 ± 0.5a</td>
</tr>
<tr>
<td>Tγδ+</td>
<td>2.5 ± 0.4</td>
<td>4.2 ± 0.2*</td>
<td>3.9 ± 1.1</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>NK+</td>
<td>1.7 ± 0.2</td>
<td>3.0 ± 0.2*</td>
<td>2.3 ± 0.4</td>
<td>3.8 ± 1.4</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. *Different from control, P < 0.05. SEB-treated group means without a common letter differ, P < 0.05.

2 IEL are expressed as cell number per linear millimeter.
populations in the intraepithelial compartment was minor; only the diet containing SDAP reduced the effects of SEB on activated T lymphocytes.

In animals challenged with enterotoxigenic E. coli, Niewold et al. (24) showed that SDAP reduced diarrhea by preventing the action of bacterial heat labile toxin. This indicates that the plasma dietary supplement contains endogenous antitoxin antibodies. However, in the model used in the present study, inflammation was induced by intraperitoneal administration of the enterotoxin. Consequently, preventative effects are less specific. For example, they may involve reducing the activity of other toxins in the lumen, and lessening the synergic effects of SEB and exogenous (luminal) enterotoxins.

In summary, the effects of SDAP on the lymphocyte populations of the lamina propria in SEB-administered rats suggest that plasma proteins can modulate the degree of activation of the immune response of diffuse GALT. SDAP was more effective than IgC, which suggests that SDAP components strengthen the effects of Ig. These effects, together with previous observations on organized GALT and mucosal integrity, support the hypothesis that plasma protein supplements protect GALT from possible excessive activation provoked by major stimulating agents such as bacterial superantigens. These effects further support the hypothesis that plasma proteins modulate the immune response at the mucosal level during an intestinal infection.

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Literature Cited