Spray-Dried Animal Plasma Prevents the Effects of Staphylococcus aureus Enterotoxin B on Intestinal Barrier Function in Weaned Rats

Anna Pérez-Bosque, Concepció Amat, Javier Polo, Joy M. Campbell, Joe Crenshaw, Louis Russell, and Miquel Moretò

Abstract

In this study, we investigated intestinal barrier function during inflammation as well as the effects of dietary supplementation with porcine spray-dried animal plasma (SDAP) proteins and porcine immunoglobulin concentrate (IC). Wistar Lewis rats were fed from d 21 (weaning) until d 34 or 35 either a control diet or a diet containing SDAP or IC. On d 30 and d 33, rats received an intraperitoneal dose of Staphylococcus aureus enterotoxin B (SEB; 0.5 mg/kg body wt; groups SEB, SEB-SDAP, and SEB-IC). SEB reduced the potential difference across the jejunum by 60%, the short-circuit current by 70%, and Na-K-ATPase activity in intestinal mucosa (all \( P < 0.05 \)). The fluxes of dextran (9 kDa) and horseradish peroxidase (HRP, 40 kDa) across the intestinal wall also increased in SEB-treated rats (\( P < 0.01, P = 0.068, \) respectively). SEB also increased HRP flux across the paracellular space (\( P < 0.05 \)). Moreover, SEB-treated rats had a reduced expression of tight junction proteins, such as ZO-1 (10% reduction; \( P < 0.05 \)) and \( \beta \)-catenin (20% reduction; \( P < 0.05 \)). Dietary supplementation with SDAP or IC prevented dextran (\( P < 0.05 \)) and HRP (\( P < 0.05 \)) paracellular flux across the intestinal epithelium. SDAP supplementation also prevented SEB effects on Na-K-ATPase activity (\( P < 0.05 \)). In our model of SEB-induced intestinal inflammation, the increased permeability across the intestinal mucosa was due to the lower expression of tight junction proteins, an effect that can be prevented by both SDAP and IC supplementation. J. Nutr. 136: 2838–2843, 2006.

Introduction

Weaning mammals are exposed to numerous stresses (e.g., changing from a liquid to a solid diet) and frequently suffer infections, mainly caused by enterotoxigenic pathogens (1). Such infections may cause intestinal inflammation, leading to high mortality rates (2). Rats, mice, and pigs can also develop intestinal inflammation during weaning, exhibiting increased plasma cytokine concentrations (3) and inflammatory T cells (4).

Increased epithelial permeability occurs during intestinal inflammation due to altered barrier function. Many toxins and inflammatory mediators are involved in this effect; e.g., Clostridium and Vibrio toxins change the localization of several tight junction proteins (5) or reduce the number of strands in tight junction (6). Moreover, toxins can also have indirect effects by inducing the release of proinflammatory cytokines such as INF-\( \gamma \) and tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)). These 2 cytokines increase epithelial permeability due to reduced ZO-1 and occludin expression (7,8).

Staphylococcus aureus enterotoxin B (SEB) belongs to the family of superantigens, which are potent polyclonal activators of the immune system that can activate a high percentage of T cells (9). SEB has the ability to disturb barrier function and ion transport, as has been described in different studies involving murine models and cell cultures (9,10). In most of these studies, proinflammatory cytokines, such as INF-\( \gamma \) and TNF-\( \alpha \), mediated the effects of SEB. In earlier experiments, SEB induced mucosal immune system activation and increased water content in feces (11). The latter may result from impaired intestinal barrier function in rats challenged with SEB.

In weanling pigs, dietary supplementation with spray-dried animal plasma (SDAP) has shown beneficial effects in both growth and performance (12). In calves infected with Cryptosporidium parvum, supplementation with proteins from bovine serum reduced fecal losses and returned villous surface area to normal values (13). SDAP has therefore been proposed as an eventual substitute for antimicrobial medication (14).

A rat model of mild intestinal inflammation was recently used to investigate the effects of dietary supplements on the pathophysiology induced by bacterial enterotoxins. This model demonstrated that dietary SDAP can modulate the immune response of gut-associated lymphoid tissue and prevent SEB-induced intestinal water secretion (11).
Thus, our starting hypothesis was that plasma protein supplementation might improve both intestinal barrier function and the intestinal transport of ions in the inflamed intestine. To analyze these possibilities in a SEB rat model, we tested the intestinal permeability to macromolecules (HRP and dextran), the expression of ZO-1 and β-catenin, and the effects directly related to ion transport.

Methods

Animals and protocols. We used male Wistar Lewis rats supplied by Harlan Ibérica. Rats were kept under stable temperature and humidity conditions, with a 12-h light/dark cycle.

All protocols used in this study were approved by the Ethical Committee of the University of Barcelona, in accordance with the regulations of the Autonomous Government of Catalonia (Departament d’Agricultura, Ramadaria i Pesca, Generalitat de Catalunya).

At d 21 after birth, rats were weaned, randomly distributed into 4 groups, housed in groups of 3, and fed experimental diets until d 34 or d 35. The studied groups included: control, rats fed a control diet and given PBS; SEB, rats fed the control diet and given SEB; SEB-SDAP, rats fed a diet supplemented with 80 g/kg spray-dried porcine plasma and treated with SEB; and SEB-IC, rats fed a diet supplemented with 22.7 g/kg porcine immunoglobulin concentrate (IC) and treated with SEB. The SDAP and IC supplements were obtained from the same batch of fresh porcine blood (APC Europe).

Pelleted diets were formulated to meet the National Research Council requirements (11). SDAP is a feed ingredient obtained after separation of RBC by centrifugation of hygienically collected blood from healthy cattle (16). The IC was obtained by purifying the immunoglobulin fraction of porcine plasma (17). The maintenance of the native structure was checked by immuno-electrophoresis and Western blotting and the activity was monitored by an ELISA specifically recognizing Escherichia coli, according to (18). Diets supplemented with SDAP or IC had similar immunoglobulin G (IgG) concentrations (14 g/kg of diet).

Intestinal inflammation was induced by the intraperitoneal injection of ketamine/xylazine. The jejunum was rapidly excised, flushed with Krebs solution, and opened lengthwise along the mesenteric line. From each rat, 3 pieces of jejunum were mounted in Ussing chambers (opening surface area of 0.63 cm²), taking care to avoid Peyer’s patches. The entire procedure was completed within 10 min and tissues were maintained under the appropriate temperature, pH, and oxygenation conditions to avoid damage. Tissues were bathed with 37°C oxygenated Krebs buffer (4 mL each side) containing (in mmol/L) 115 NaCl, 2 KH₂PO₄, 1 MgCl₂, 1.25 CaCl₂, 8 KCl and 25 NaHCO₃, pH 7.4. This buffer included 100 mmol/L mannose, 25 mmol/L l-glutamine, and 5 mmol/L β-hydroxybutyrate as an energy source. Tissues were allowed to equilibrate for 15 min before electrical variables and intestinal permeability were evaluated. Two pairs of electrodes were used to monitor the transmural potential difference (PD, in mV) and to inject a current that maintained zero PD (short-circuit current [Isc] expressed as μA/cm²). Conductance (G) was calculated according to Ohm’s law and expressed as mS/cm².

Measurement of Na+-K+-ATPase activity. The activity of the ouabain-sensitive Na+-K+-ATPase activity (Na+-K+-ATPase, EC 3.6.1.3) was measured in jejunal mucosa homogenate by spectrophotometrically determining p-nitrophenil phosphate hydrolysis, with or without ouabain (19). Protein concentration was determined using the Bio-Rad protein assay performing all assays in triplicate.

Intestinal permeability. FITC-dextran 4 kDa was used as a marker of paracellular permeability to macromolecules (20). The tracer (125 μmol/L, Sigma) was added to the mucosal buffer. Samples (100 μL) were obtained at 15-min intervals from the serosal compartment (between 60 and 120 min after dextran addition) and were replaced by fresh buffer to maintain a constant volume. Dextran concentration was measured in a fluorometer (Victor, Perkin Elmer).

Protein flux. We used horseradish peroxidase (HRP) as a protein model because its molecular mass (40 kDa) is similar to those of many antigenic food proteins (21). HRP (10 μmol/L, type IV, Sigma) was added to the mucosal buffer, obtaining samples as previously described. A kinetic assay (22) was used to measure intact HRP Microplate Reader to gauge color intensity (Multiscan, Labsystems).

Electron microscopy. To examine the route of antigen uptake across the intestinal epithelium, some tissues were removed from Ussing chambers following a 20 min of incubation in the presence of HRP. Tissues were processed as described by Kiliaan et al. (23). Images were taken by a 1010 transmission electron microscope (JEOL). To obtain information on the uptake and pathway of HRP transport across the epithelium, we evaluated 4 high-power electron photomicrographs per rat. The amount of HRP in the paracellular space was quantified using the NIH image program (24) and expressed as the mean pixel intensity.

Immunohistochemistry of ZO-1 and β-catenin. The jejunum was excised and the immunolocalization was done as described in Garriga

| TABLE 1 | Electrical variables of jejunum from control, SEB, SEB-SDAP, and SEB-IC rats1 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Conductance, mS/cm² | Potential difference, mV | Short circuit current, μA/cm² | Na-K-ATPase activity, nkat/mg protein |
| Control | 21.8 ± 1.1 | 3.6 ± 0.6 | 78.4 ± 11.8 | 0.22 ± 0.02 |
| SEB | 18.2 ± 1.9 | 1.4 ± 0.3* | 23.0 ± 4.2* | 0.15 ± 0.01* |
| SEB-SDAP | 15.7 ± 1.3 | 2.7 ± 0.6 | 43.4 ± 12.3 | 0.20 ± 0.02 |
| SEB-IC | 15.3 ± 1.2 | 1.9 ± 0.7 | 35.4 ± 12.1 | 0.19 ± 0.03 |

1 Values are means ± SEM, n = 7–10 animals. Symbols indicate significant differences, P < 0.05: *vs. control group; †vs. SEB group.

Figure 1 Dextran flux (A) and HRP flux (B) across the intestinal wall in control, SEB, SEB-SDAP, and SEB-IC rats. Results are means ± SEM, n = 8–10. Symbols indicate significant differences, P < 0.05: *vs. control group; †vs. SEB group.
et al. (19). The primary antibodies used were as follows: rabbit polyclonal anti-ZO-1 and a mouse monoclonal anti-β-catenin (1/125, Zymed Laboratories and 1/250, Becton Dickinson, respectively). The secondary antibodies included Alexa Fluor 488 conjugated goat anti-mouse antibody and Alexa Fluor 488 conjugated goat anti-rabbit antibody (1/300, Molecular Probes). To stain the cytoskeleton, we incubated slides with Alexa Fluor 633 conjugated phalloidin (1/75, Molecular Probes). Negative controls were performed without the primary antibodies and samples remained stored at 4°C until observation with a confocal microscope (CLSM SPII, Leica).

Image acquisition and processing. We analyzed the captured images using the NIH image program (24). To quantify the fluorescence associated with ZO-1 and β-catenin proteins, we selected the tight junction area and adhesion belt by colocalization with actin stain.

Statistical analyses. Samples that were analyzed in duplicate or triplicate were treated as single values using the arithmetic mean of replicates. Results are given as the means ± SEM of 7–10 rats. To analyze the effects of enterotoxin administration, we compared SEB rats with control rats by ANOVA using SPSS-11.0 software. To study the effect of dietary supplementation on our intestinal inflammation model, we compared rats fed the supplemented diets (SEB-SDAP, SEB-IC) with those fed the nonsupplemented diet (SEB) using another ANOVA followed by Scheffé post hoc test. Time effects were analyzed using a 2-way ANOVA. Associations were assessed using Pearson’s correlation test. Differences were considered significant at \( P < 0.05 \).

Results

Electrical variables. Neither SEB administration nor diet supplementation modified intestinal baseline conductance (Table 1). Intestinal conductance was monitored as a tissue viability marker (23). The stability of tissue conductance over time in all groups (i.e., the absence of any significant difference between the initial...
values and those at the end of the experiment) indicated that tissue deterioration during the 2-h experimental period was negligible.

SEB treatment reduced the PD by 60% \( (P < 0.01; \text{Table 1}) \).

SDAP supplementation tended to prevent this reduction \( (P = 0.077) \), whereas SEB-administered rats fed the IC supplement did not differ from other groups. SEB treatment also reduced Isc by 70% \( (P < 0.01; \text{Table 1}) \); supplemental SDAP and IC did not modify the effects of SEB.

**Na-K-ATPase activity.** SEB administration reduced the activity of Na-K-ATPase by 30% \( (P = 0.05, \text{Table 1}) \), and SDAP supplementation prevented SEB-induced reduction \( (P < 0.05) \). The activity in the SEB-IC group did not differ from those of the other 3 groups.

**Intestinal paracellular permeability.** SEB treatment induced a 20% increase in FITC dextran flux across the intestinal wall \( (P < 0.05; \text{Fig. 1A}) \). Both supplemented diets prevented the SEB-induced increase in dextran flux \( (P < 0.01 \text{ and } P < 0.05) \).

**HRP flux.** SEB administration tended to enhance the HRP movement across the mucosa \( (40\%; P = 0.068, \text{Fig. 1B}) \). Dietary supplementation with SDAP \( (P = 0.055) \) and IC \( (P = 0.077) \) tended to prevent the effects of SEB on HRP flux.

There was a significant positive correlation between dextran and HRP flux values \( (r = 0.993, P < 0.001) \), suggesting that the paracellular route contributes to increased protein translocation across the intestine as induced by SEB administration.

**Expression of ZO-1.** The fluorescence intensity from ZO-1 immunostaining was weaker in SEB-treated rats than in control rats at 24 h and 48 h following SEB administration \( (\text{Fig. 2A}) \). Quantification of fluorescence intensity showed that SEB-treated rats exhibited a considerably lower fluorescence intensity 24 h after the second dose \( (P < 0.01; \text{Fig. 2B}) \). Furthermore, 48 h later, the fluorescence intensity associated with the expression of ZO-1 in the SEB group was still lower than in the control group \( (P < 0.01) \). None of the supplemented diets modified the effects induced by superantigen treatment at either time.

ZO-1 expression at 24 h after SEB administration tended to be negatively correlated with dextran flux \( (r = -0.6; P = 0.087) \), suggesting that increases in dextran flux are paralleled by reductions in ZO-1 expression at the epithelial level.

**Expression of β-catenin.** SEB-treated rats showed lower fluorescence intensity for β-catenin staining than did control rats at both 24 and 48 h \( (\text{Fig. 3A,B}) \). The effect at 24 h after the second SEB-dose \( (P < 0.01; \text{Fig. 3B}) \) tended to be greater than that at 48 h after the second dosing \( (P = 0.06) \). When inflamed

---

**TABLE 2** Relative HRP abundance in the paracellular spaces of small intestine epithelium from control, SEB, SEB-SDAP, and SEB-IC rats \( ^1 \)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean pixel intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>SEB</td>
<td>119 ± 6*</td>
</tr>
<tr>
<td>SEB-SDAP</td>
<td>97 ± 4†</td>
</tr>
<tr>
<td>SEB-IC</td>
<td>99 ± 4</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM, \( n = 4 \). Symbols indicate significant differences \( (P < 0.05) \):

*vs. control group; †vs. SEB group.
rats were fed the supplemented diets, the expression of β-catenin at both time points did not differ from that of SEB rats.

β-Catenin expression tended to be negatively correlated with dextran flux (r = −0.88; P = 0.087). Furthermore, β-catenin expression was positively correlated with that of ZO-1 (r = 0.84; P < 0.05). These results suggest that the increase in dextran flux induced by SEB treatment is associated with a reduction in the tightness of the epithelial junctional complex.

**Electron photomicroscopy.** We did not observe epithelial damage in the tissues of any rats (Fig. 4). Moreover, in control rat tissues, there was no HRP in the paracellular spaces between adjacent epithelial cells (Fig. 4A; Table 2). In contrast, photomicrographs of most SEB-treated rats revealed HRP within paracellular spaces, located just below the tight junctions (Fig. 4B). This effect, induced by SEB treatment, was prevented by SDAP (P < 0.05) and IC (P = 0.056) supplementation (Table 2).

SEB-treated rats showed very few small endosomes containing HRP (Fig. 4F), suggesting that the transcellular pathway of HRP must be very low and therefore cannot explain the increased HRP flux induced by SEB. Inflamed rats fed supplemented diets did not show any endosomes, while tissue appearance under electron microscopy was similar to that of control rats (Fig. 4G,H,E, respectively). For these reasons, the total number and density of endosomes could not be quantified.

**Discussion**

SDAP and IC are dietary supplements given to farm animals to improve their growth and performance during the postweaning period. Weaning mammals exhibit an increased susceptibility to infectious diseases and diarrhea, which in turn raises mortality rates (1). Previous studies using Lewis rats in a model of intestinal inflammation using SEB showed that plasma protein supplements can prevent reductions in nutrient absorption (19), improve intestinal immune response, and reduce water secretion (11). We therefore deemed it relevant to determine not only whether SEB-induced intestinal inflammation was paralleled by alterations in intestinal barrier function, but also whether SDAP or IC supplementation could ameliorate these effects.

Rats administered with SEB showed increased paracellular permeability to macromolecules, which was associated with a reduction in epithelial tightness observed in the reduced expression of ZO-1 (in tight junction) and β-catenin (in adherent junction). Similar effects were described in a mouse model of intestinal inflammation induced by the same superantigen (9). Proinflammatory cytokines are the main candidates for mediating these effects because they are induced by SEB (9,25) and facilitate antigen flux across the paracellular space (26).

Consistent with the above observations, toxins like SEB stimulate lymphocytes to secrete INF-γ and TNF-α, which can either disassemble tight junction proteins (27) or reduce their expression (7,8). The resulting increased intestinal permeability can enhance local inflammation, as happens in ulcerative colitis and inflammatory bowel disease (28).

The flux of HRP across the intestinal wall has also been studied. HRP is widely used as a tracer of mucosal permeability to food antigens (21). SEB treatment increases HRP intestinal flux, suggesting that there is higher luminal permeability to macromolecules during intestinal inflammation. Moreover, in the inflammatory model used in the present study, there is a positive correlation between dextran and HRP fluxes, indicating that the paracellular route is the main factor increasing the transmural protein movement induced by experimental inflammation (23).

Using electron microscopy, we confirmed the principal contribution of the paracellular pathway, showing not only that HRP crosses it to reach the serosal side of the epithelium, but also that the contribution of the transcellular pathway is negligible. In the present study, the HRP flux across the epithelium in both control and SEB-treated rats is very low, probably reflecting the low degree of mucosal damage that characterizes the current SEB model protocol, as previously shown (19).

SEB reduced both the PD and the ISC across the intestine, consistent with results obtained in other gastrointestinal inflammatory models (29). We hypothesize that in SEB-treated rats, there is a reduction in intestinal sodium transport, because similar phenomena had been described for suckling rats infected with *C. parvum* (30). This hypothesis was supported by the reduction in Na-K-ATPase activity in the intestinal mucosa of SEB-treated rats. The absence of any SEB effects on tissue conductance, despite its effects on junctional protein expression, indicates that changes in permeability markers are not always correlated with electrical parameters, as shown by Balda et al. (31). Proinflammatory cytokines, such as INF-γ and TNF-α, reduce the expression of the Na-K-ATPase α-subunit as well as the activity of the sodium pump (32,33), which would account for the changes in certain intestinal electrical parameters observed in the present study.

SDAP and IC supplementation reduced the effects of SEB on dextran and HRP paracellular flux. The resulting reduction in permeability may prevent the passage of microbial and food antigens to the interstitial space, thereby blocking local inflammation (34). This pattern, together with our observations that SDAP supplementation can attenuate the effects of SEB on intestinal immune activation (11), are consistent with the hypothesis that SDAP can reduce the release or activity of local intestinal proinflammatory mediators (12).

We conclude that dietary supplementation with plasma proteins (SDAP and IC) can in part prevent alterations in epithelium structure during experimental inflammation, thereby improving intestinal mucosal barrier function.

**Acknowledgments**

The authors gratefully acknowledge the technical assistance of Ms. Lluïsa Miró. The valuable help of the staff of the Confocal Microscopy and Electron Microscopy Services of the University of Barcelona is also acknowledged.

**Literature Cited**

5. Chen ML, Pothoulakis C, LaMont JT. Protein kinase C signaling regulates ZO-1 translocation and increased paracellular flux of T84 colonocytes exposed to Clostridium difficile toxin A. J Biol Chem. 2002;277:4247–54.
6. Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S. Clostridium perfringens enterotoxin fragment removes speci-


