Damage to the Intestinal Epithelial Barrier by Antibiotic Pretreatment of Salmonella-Infected Rats Is Lessened by Dietary Calcium or Tannic Acid

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Abstract

Perturbation of the intestinal microbiota by antibiotics predisposes the host to food-borne pathogens like Salmonella. The effects of antibiotic treatment on intestinal permeability during infection and the efficacy of dietary components to improve resistance to infection have not been studied. Therefore, we investigated the effect of clindamycin on intestinal barrier function in Salmonella-infected rats. We also studied the ability of dietary calcium and tannic acid to protect against infection and concomitant diarrhea and we assessed intestinal barrier function. Rats were fed a purified control diet including the permeability marker chromium EDTA (CrEDTA) (2 g/kg) or the same diet supplemented with calcium (4.8 g/kg) or tannic acid (3.75 g/kg). After adaptation, rats were orally treated with clindamycin for 4 d followed by oral infection with Salmonella enteritidis. Two additional control groups were not treated with antibiotics and received either saline or Salmonella. Urine and feces were collected to quantify intestinal permeability, diarrhea, cytotoxicity of fecal water, and Salmonella excretion. In addition, Salmonella translocation was determined. Diarrhea, CrEDTA excretion, and cytotoxicity of fecal water were higher in the clindamycin-treated infected rats than in the non-clindamycin–treated infected control group. Intestinal barrier function was less in the Salmonella-infected rats pretreated with antibiotics compared with the non-clindamycin–treated rats. Both calcium and tannic acid reduced infection-associated diarrhea and inhibited the adverse intestinal permeability changes but did not decrease Salmonella colonization and translocation. Our results indicate that calcium protects against intestinal changes due to Salmonella infection by reducing luminal cytotoxicity, whereas tannic acid offers protection by improving the mucosal resistance.

Introduction

The use of antibiotics is ubiquitous in our present society. The use of broad-spectrum antibiotics like clindamycin disturbs the indigenous microbiota in the intestine. This perturbation of the intestinal microbiota predisposes the host to enteric pathogens such as Salmonella (1). Eradication or suppression of parts of the indigenous microbiota can decrease the colonization resistance of the host (2). For example, competition for mucosal adhesion sites (3) and growth substrates (4) can decrease and thus facilitate intestinal colonization of bacterial pathogens.

Invasive species, like certain types of Salmonella, are able to translocate from the intestinal lumen to extra-intestinal organs (5,6), a phenomenon which is often accompanied by increased mucosal paracellular permeability and diarrhea (7). The magnitude of these latter events not only depends on the amount of pathogens present but is also strongly determined by the intestinal defense status of the host (7).

Diet may be a tool to strengthen this intestinal defense or resistance to infection and infectious diarrhea. We previously showed that dietary calcium enhances intestinal resistance to infectious disease and protects rats (8–11) against Salmonella and enterotoxigenic Escherichia coli associated disease. The protective effects of calcium are associated with an increase of lactobacilli in the microbiota (8,10). Whether this increase is causally related to the protective effect of calcium is not known yet but can be studied by using antibiotics that lower indigenous lactobacilli.

A group of dietary compounds with the potential to inhibit intestinal infections are the polyphenols. For example, complex
(hydrolysable) tannins, which are widespread in some vegetable foods such as grapes, berries, and the wines produced from them (12), have antimicrobial effects against Gram-negative bacteria (13). The polyphenol tannic acid is a complex hydrolysable tannin. It is composed of 6–9 gallic acid molecules esterified with glucose and is used as a preservative by the food industry (14, 15).

In this study, we investigated the effect of clindamycin on Salmonella infection, translocation, and intestinal permeability using a rat model. Second, we examined whether dietary calcium is still able to protect against intestinal infection when the indigenous microbiota is temporarily crippled. Third, protective effects of tannic acid were investigated in the same model.

Methods

Experiment approval and rats. The animal experiment was approved by the animal welfare committee of Wageningen University (Wageningen, The Netherlands). Specific pathogen-free male Wistar rats (WU, Harlan), 8 wk old at the start of the dietary intervention, were housed individually in metabolic cages. The animal room was temperature (20–21°C) and humidity (50–60%) controlled with a 12-h-light/12-h-dark cycle.

Diets, clindamycin treatment, and infection. Rats were fed a purified control diet as previously described (7) containing (per kg) 200 g acid casein, 326 g cornstarch, 174 g glucose, 160 g palm oil, 40 g corn oil, 50 g cellulose, and vitamin and mineral mix according to AIN-93 (except for calcium) (16). To mimic the composition of a Western human diet, the control diet was supplemented with either calcium-[(antibiotic-treated infected rats fed the calcium diet (AICa)] or tannic acid-[(antibiotic-treated infected rats fed the tannic acid diet (AITa)]. Two additional groups (n = 8/diet) that received clindamycin plus Salmonella was temporarily crippled. Third, protective effects of tannic acid were investigated in the same model.

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To investigate serum IgM specific to *Salmonella*, a maxisorp 96-well plate (Nunc) was coated with 135 μL of a heat-killed *S. enteritidis* suspension. This suspension originally contained 10^10 CFU/mL *S. enteritidis*, identical to the strain used in the infection study, in PBS (pH 7.4), which had been incubated at 60°C for 90 min. After incubating the plates for 20 h at 4°C, the wells were blocked with 4% Protifar plus (Nutricia) in PBS for 1 h. Plates were incubated with 100 μL of diluted serum for 2 h and with 100 μL HRP-mouse anti-rat IgM (Invitrogen) for 1.5 h at room temperature. Between all incubations, the plates were washed thoroughly with PBS 0.05% Tween-20. Finally, 100 μL of a reaction mixture containing hydrogen peroxide and o-phenylenediamine was added. The reaction was discontinued after 20 min by adding 100 μL 1 mol/L H2SO4 and the absorbance was read at 490 nm on an automated ELISA plate reader (Dynatech MR7000; Trade Tek). Negative controls showed that the HRP-mouse anti-rat IgM did not bind to wells coated with the blocking solution only.

**Statistical analysis.** All data are expressed as means ± SEM and statistics were performed using Graphpad Prism 5 software (GraphPad Software). Our aim was to investigate the effect of clindamycin on infection and the dietary effects in clindamycin-pretreated infected rats. Therefore, the predefined comparisons of interest were: UC and IC vs. AIC, and AICa and AITa vs. AIC. In addition, the effect of clindamycin on the fecal microbiota in rats fed the different diets was investigated prior to infection.

Data were tested for normality by using the Kolmogorov-Smirnov and Shapiro-Wilk tests. If normally distributed, differences were tested for significance using 1-way ANOVA and, when needed, were followed by Dunnett’s post hoc test for comparisons to the AIC group. Within-group comparison of the microbiota data before and after clindamycin treatment was tested by a paired t test or Wilcoxon’s matched paired test for data with equal and unequal variances, respectively. *Salmonella* output in feces was determined at multiple time points and therefore these data were analyzed by repeated-measures 2-way ANOVA (mixed-model) followed by Bonferroni’s post testing for comparisons to the AIC group. Differences were considered significant when *P* < 0.05.

**Results**

**Growth and food intake.** At the start of the study, the body weight of the rats was 279 ± 1.3 g. Food intake prior to clindamycin treatment was 20 ± 0.3 g/d in all groups (data not shown). Daily food intake postinfection or sham treatment was higher in the IC and UC groups (17 ± 0.3 g/d) than in the AIC group (14 ± 0.3 g/d; *P* < 0.05). Daily food intake did not differ from the AIC group in the AICa and AITa groups (data not shown).

Prior to infection, body weight gain was 4 ± 0.1 g/d in all groups. Postinfection, body weight gain in all clindamycin-treated groups was 1 ± 0.3 g/d, whereas non-clindamycin-treated rats gained more weight (3 ± 0.2 g/d; *P* < 0.05). Body weight gain did not differ from the AIC group in either the AICa or AITa group (data not shown).

**Lactobacilli and enterobacteria in feces.** The effects of clindamycin and the diets on the intestinal microbiota were determined prior to infection. In line with our earlier studies (10), fecal lactobacilli were higher and enterobacteria were lower in the AICa group than in the AIC group (*P* < 0.05) (Table 2). The enterobacteria were also lower in the AITa group than in the AIC group prior to clindamycin treatment (*P* < 0.05) (Table 2).

Clindamycin drastically reduced the number of lactobacilli in all groups (*P* < 0.05) (Table 2), whereas enterobacteria increased in AICa and AITa groups compared with preclindamycin levels, resulting in levels that were identical to the AIC group. This showed that dietary effects on the gut microbiota were abolished after clindamycin treatment, except for slightly more lactobacilli in the AICa group compared with the AIC group (*P* < 0.05) (Table 2).

**Salmonella colonization.** Clindamycin treatment clearly decreased colonization resistance of the intestine, because fecal *Salmonella* excretion was 100-fold higher in the AIC group than in the IC group (*P* < 0.05) (Fig. 1). Fecal *Salmonella* excretion was higher in the AIC group than in the IC group at each day after infection. Colonization levels were not lower in the AICa and AITa groups compared with the AIC group. At d 3, the AITa group was higher than the AIC group (*P* < 0.05) (Fig. 1).

**Salmonella translocation.** *Salmonella* translocation to MLN, liver, and spleen was lower in the IC group than in the AIC group (*P* < 0.05) (Table 3) and neither the AICa group nor the AITa group differed from the AIC group. Results of *Salmonella* culture of extra-intestinal organs were confirmed by analysis of urinary NOx excretion. The AUC for infection-induced urinary NOx was higher in the AIC group than in the UC and IC groups (*P* < 0.05) (Table 3). The levels in the AICa and AITa groups did not differ from the AIC group. Urinary NOx did not differ among the 5 groups before or after clindamycin treatment (data not shown), indicating that neither the diets nor a drastic change in the microbiota by clindamycin treatment affected NOx excretion.

**Diarrhea.** Fecal relative wet weight was determined to quantify possible treatment effects on diarrhea. Clindamycin did not significantly affect fecal wet weight in the period prior to infection (data not shown). In contrast, fecal wet weight was greater in the AIC group than the IC group (*P* < 0.05) (Table 4). Infection-induced diarrhea was less in the AICa and AITa groups than the AIC group (Table 4). This indicated that despite similar *Salmonella* colonization and translocation levels, the normal functioning of the surface epithelium was better maintained in the rats fed the calcium or tannic acid diet. Cytotoxicity of fecal water, which can affect the surface epithelium, was higher in the AIC group than in the UC and IC groups (*P* < 0.05) (Table 4). This cytotoxicity in the AITa group did not differ from the AIC group but was much lower in the AICa group compared with the AIC group (*P* < 0.05) (Table 4).

**Intestinal permeability.** The barrier function of the epithelial monolayer was investigated by measuring urinary recovery of

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Effect of dietary calcium, tannic acid, and clindamycin on viable lactobacilli and enterobacteria in the indigenous microbiota of rats before and after clindamycin treatment and prior to infection1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Lactobacilli (log CFU/g)</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Before</td>
<td>AIC</td>
</tr>
<tr>
<td></td>
<td>AICa</td>
</tr>
<tr>
<td></td>
<td>AITa</td>
</tr>
<tr>
<td>After</td>
<td>AIC</td>
</tr>
<tr>
<td></td>
<td>AICa</td>
</tr>
<tr>
<td></td>
<td>AITa</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8. *Different from AIC, P < 0.05; **different from the before-clindamycin period, P < 0.05.

Clindamycin-stressed mucosal barrier
oral CrEDTA, which is a marker of paracellular permeability in gut epithelium (7). CrEDTA recovery almost doubled in the AIC group compared with the UC and IC groups (Table 4). However, this permeability was lower in the AICA and AITA groups compared with the AIC group (P < 0.05) (Table 4).

Whether differences in intestinal permeability affected the leakage of noxious luminal compounds was studied by measuring the antibody response to core endotoxin. The IgM response to core endotoxin was higher in the AIC group compared with the IC group (P < 0.05) (Table 4). Both AICA and AITA groups had a lower IgM response compared with the AIC group (P < 0.05). The difference in these IgM levels could be caused by modulation of the immune response by calcium and tannic acid. Therefore, we also measured serum levels of IgM directed against Salmonella. This IgM response did not differ significantly among the AICA and AITA groups compared with the AIC group (data not shown), a result in line with the comparable translocation levels of this bacterium. Thus, these results indicated that the effects of calcium and tannic acid on core endotoxin IgM levels were not due to a generic modulation of the immune response (Table 4).

Discussion

This study corroborates earlier findings that antibiotic treatment predisposes the host to Salmonella infection (1,2). We now show that this phenomenon is accompanied by diarrhea, increased intestinal permeability, and an increased IgM response to core endotoxin in serum. These symptoms are associated with a major increase in the cytotoxicity of fecal water, indicating increased irritating effects of the luminal content on the surface epithelium.

The present study indicates that Salmonella colonization levels were not lower in the AICA group than in the AIC group. This is in contrast to results in a conventional infection model in which reduced colonization by dietary calcium is associated with an increase in fecal lactobacilli and a decrease in the number of enterobacteria (10). These effects of dietary calcium were present in the AICA group compared with the AIC group prior to clindamycin treatment. After clindamycin treatment, these presumed beneficial changes of the intestinal microbiota were almost completely abrogated. Except for slightly more lactobacilli in the AICA group, levels from all groups were identical to the AIC group. The suppression of enterobacteria in the AICA group compared with the AIC group was abolished by clindamycin, which can be explained by the fact that clindamycin favors growth of these bacteria (22). These results support our hypothesis (10) that calcium improves the resistance to Salmonella via stimulation of the competitive microbiota. They falsify, however, our alternative hypothesis (11) that protection is due to binding of Salmonella to amorphous calcium phosphate in the intestinal lumen. This proposed binding should not be affected by the clindamycin treatment and then colonization levels in the AICA group would have been lower than in the AIC group.

Despite the absence of an inhibitory effect on Salmonella colonization and translocation, the infection-related diarrhea was less in the AICA group than in the AIC group. This indicated that, despite similar Salmonella colonization and translocation levels, the normal functioning of the surface epithelium was better maintained in the AICA group than in the AIC group. The surface epithelium is highly affected by the cytotoxicity of fecal water (23). The present study provides evidence that clindamycin greatly increased the cytotoxicity of fecal water, because levels were much higher in the AIC group than in the IC group. Previous animal (9) and human (20,24) studies have shown that calcium precipitates irritating bile acids and other cytotoxic surfactants and thus reduces cytotoxicity of luminal contents, which prevents epithelolysis and likely preserves intestinal barrier function. These human studies used a dietary calcium dosage within the same concentration range as in the present study. Therefore, the proposed protective mechanism of calcium in rats is likely to be of relevance to humans.

Here, we present the novel finding that dietary calcium preserved the barrier function in a sensitized Salmonella infection model, because leakage of CrEDTA from the intestinal

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>MLN</th>
<th>Spleen</th>
<th>Liver</th>
<th>Urinary NOx</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC</td>
<td>nd[^2]</td>
<td>3.20 ± 0.10</td>
<td>2.13 ± 0.12^*</td>
<td>61 ± 11^*</td>
</tr>
<tr>
<td>IC</td>
<td>4.66 ± 0.11^*</td>
<td>nd</td>
<td>2.32 ± 0.14</td>
<td>350 ± 36</td>
</tr>
<tr>
<td>AIC</td>
<td>5.01 ± 0.09</td>
<td>3.28 ± 0.14</td>
<td>3.23 ± 0.19</td>
<td>300 ± 34</td>
</tr>
<tr>
<td>AICA</td>
<td>5.05 ± 0.13</td>
<td>3.37 ± 0.13</td>
<td>2.85 ± 0.15</td>
<td>295 ± 35</td>
</tr>
<tr>
<td>AITA</td>
<td>4.97 ± 0.11</td>
<td>3.39 ± 0.11</td>
<td>3.00 ± 0.16</td>
<td>295 ± 35</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8 except UC, n = 5. *Different from AIC, P < 0.05.
2 Not determined.
3 Data were corrected for baseline values.

### Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Relative fecal wet weight</th>
<th>Cytotoxicity</th>
<th>Urinary CrEDTA excretion</th>
<th>IgM to core endotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC</td>
<td>68.5 ± 2.2^*</td>
<td>22.2 ± 4.2^*</td>
<td>3.6 ± 0.3^*</td>
<td>0.37 ± 0.11^*</td>
</tr>
<tr>
<td>IC</td>
<td>72.7 ± 1.8^*</td>
<td>27.6 ± 2.9^*</td>
<td>4.2 ± 0.4^*</td>
<td>0.33 ± 0.04^*</td>
</tr>
<tr>
<td>AIC</td>
<td>78.5 ± 0.7</td>
<td>81.9 ± 5.7</td>
<td>7.2 ± 0.9</td>
<td>1.05 ± 0.13</td>
</tr>
<tr>
<td>AICA</td>
<td>71.9 ± 1.0^*</td>
<td>40.7 ± 7.8^*</td>
<td>4.1 ± 0.4^*</td>
<td>0.60 ± 0.10^*</td>
</tr>
<tr>
<td>AITA</td>
<td>71.2 ± 1.0^*</td>
<td>39.3 ± 3.5</td>
<td>3.8 ± 0.4^*</td>
<td>0.67 ± 0.07^*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8 except UC, n = 5. *Different from AIC, P < 0.05.
Because cytotoxicity of fecal water was not lower in the AICa group. Increased paracellular permeability can facilitate translocation of noxious bacterial components such as lipopolysaccharide (LPS), the outer membrane component of Gram-negative bacteria. Bacterial translocation of Salmonella bacteria itself occurs via a transcellular route. This transport is facilitated by a mechanism that involves binding to epithelial cells by the type III secretory system, which is present on the bacterium (25,26). This type III secretory system allows direct activation of components of the host cytoskeleton by dedicated bacterial effectors (5). Translocation of Salmonella is not affected by the difference in cytotoxicity. Colonization levels were identical in the AIC and AICa groups, but cytotoxicity was clearly higher in the AIC group. Despite the difference in cytotoxicity translocation, levels of Salmonella were identical in both groups. This shows that increased luminal cytotoxicity did not affect Salmonella translocation in our study. Furthermore, we investigated the antibody response directed to core endotoxin, which is the conserved part of bacterial LPS. Increased serum LPS is associated with increased paracellular permeability, e.g. induced by nonsteroidal antiinflammatory compounds (27,28). The IgM response to core endotoxin was significantly lower in the AICa group than in the AIC group, a result in line with intestinal permeability differences between these groups. This indicated that dietary calcium inhibited the leakage of LPS. We speculate that this is due to the observed inhibition of luminal cytotoxicity (Table 4). Thus, calcium lowers the concentration of cytotoxic surfactants to which the mucosa is exposed and this may inhibit paracellular permeability, as discussed above. This protective effect of calcium on leakage of noxious luminal components is further supported by our recent finding that dietary calcium ameliorates colitis in a transgenic rat model of inflammatory bowel disease (29). On the other hand, the observed difference in the luminal calcium concentration due to our dietary intervention might affect gut barrier function more directly. Obviously, alterations in tight junction proteins as the primary regulators of intestinal permeability are expected. Recent studies indicate that calcium did not induce changes in gene expression of tight junction proteins (e.g. claudins, occludin, zona occludens, and myosin IXb) (29), although morphological and structural alterations in tight junctions might exist. For example, internalization into the cellular cytoplasm and phosphorylation of tight junction proteins are suggested to be important for leakage of the mucosal barrier (30). Extracellular (luminal) calcium is crucial for maintenance of intestinal tight junction function in cell studies. However, these effects seem to occur at free calcium concentrations up to 0.1 mmol/L (31), which is at least 50 times lower than the luminal calcium levels of rats fed the control or calcium diet (29). Therefore, direct effects of luminal calcium on tight junction proteins in vivo seem less likely. Despite antimicrobial effects of (hydrolysable) tannins like tannic acid in vitro (13,15), neither intestinal Salmonella colonization nor translocation was lower in the AITa group than in the AIC group. This indicated that possible antimicrobial effects of tannic acid were overruled by the clindamycin pretreatment, as shown by our culture data (Table 2; Fig. 1). Notwithstanding that, tannic acid, although chemically unrelated to calcium, had similar effects on diarrhea and epithelial permeability in the AITa group compared with the AIC group. Because cytotoxicity of fecal water was not lower in the AITa group than in the AIC group, this polyphenol apparently maintained the proper functioning of the mucosa by increasing its resistance to luminal cytotoxicity. At present, we can only speculate about the mechanism of this unexpected barrier-strengthening effect of tannic acid. As a first hypothesis, we propose that protection is due to direct binding of tannic acid to the outer leaflet of the apical membrane of the surface epithelium, as is shown for ligated intestinal segments of rats (32). We conjecture that a surface layer of bound tannic acid polymers prevents the interaction of hydrophobic, and thus cytotoxic, surfactants with the apical membrane. This is supported by the finding that topically applied tannic acid protects human skin epithelium against hydrophobic surfactants such as laurel sulfate (33). As an additional mechanism, we propose that gallic acid, taken up by enterocytes, may have additional protective effects. This phenolic monomer is released from the polymeric tannic acid by hydrolysis and fermentation by intestinal bacteria (34). A recent in vitro study (35) showed that gallic acid, taken up by mast cells, lowers cytosolic calcium and inhibited stress signaling via protein kinases by preventing nuclear factor-κ B activation. This is in line with another study (36) that showed that gallic acid inhibits several protein kinases by competitive inhibition of the ATP binding site. Decreasing cytosolic calcium and protein kinase activity lowers fluid and ion secretion, such as chloride secretion regulated by the cystic fibrosis transmembrane conductance regulator, by enterocytes (37). Because activation of myosin light-chain kinase increases tight junction permeability (38), we speculate that this can be counteracted by gallic acid. These 2 hypothetical mechanisms are difficult, if not impossible, to study in vivo. Therefore, we plan further in vitro studies in which enterocytes, cultured in a Transwell system, are apically exposed to cytotoxic surfactants in the absence and presence of tannic acid or gallic acid. It should be noted that the suggested mechanism by changes in cytosolic calcium is not relevant to our calcium intervention. A diet high in calcium does increase luminal calcium levels, but it does not increase intracellular calcium levels. Systemic (serum) calcium concentrations are tightly regulated, and thus stable, and are not influenced by dietary calcium supplementation (39). Therefore, the effects of an increase in intracellular calcium differ from the effects of increased luminal calcium. It is important to extrapolate our dietary interventions to the human situation. The calcium content of the calcium-supplemented diet was 4.8 g/kg dry food, which corresponds to a daily calcium intake of 2.4 g in humans, assuming that humans have a daily dry food intake of ~500 g. In general, human dietary calcium intake in the Western world is ~1.4 g/d (24). This indicates that the calcium-supplemented diet provided more than the general habitual dietary calcium intake. However, this is not an unrealistic intake when using calcium supplements. Similarly, it can be calculated that the tannic acid diet of 3.75 g/kg dry food used in this study corresponds to a daily tannic acid intake of 1.9 g in humans. The estimated daily tannic acid intake in humans (in the US) is 1 g (40). Therefore, the applied tannic acid diet can also be extrapolated to humans using tannic acid supplements. In conclusion, clindamycin resulted in increased diarrhea, intestinal permeability, and elevated IgM levels to core endotoxin in a Salmonella infection model. These symptoms were associated with increased cytotoxicity of fecal water. Both calcium and tannic acid lessened these effects but without decreasing Salmonella colonization and translocation. Further investigations should focus on the mechanisms by which these dietary components maintain proper barrier function of the surface epithelium in this mucosal stress model. Our results indicate that calcium protects by reducing luminal cytotoxicity.
and that tannic acid functions by improving the mucosal resistance to luminal cytotoxicity. Verification of the human relevance of our findings in exploratory studies, e.g. in antibiotic-treated humans, is also needed.

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Literature Cited


