Zinc Deficiency Suppresses the Development of Oral Tolerance in Rats

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ABSTRACT Oral tolerance is a specific immune unresponsiveness to food antigens to prevent hypersensitivity reactions. We investigated whether zinc deficiency affects oral tolerance. Rats were fed a control (C) or zinc-deficient (ZD) diet, or pair-fed (PF) to ZD rats for 28 d. Beginning on d 7, rats were administered ovalbumin (OVA) orally to induce tolerance, or PBS 3 times/wk, and were then immunized by OVA injection. The proliferation of mesenteric lymph node (MLN) and spleen lymphocytes after in vitro OVA stimulation and the delayed-type hypersensitivity were higher in OVA-fed ZD than in OVA-fed C rats and not different between OVA- and PBS-fed ZD rats, indicating a suppression of tolerance. Lymphocyte proliferation did not differ between PF and C rats. Expressions of cytokines involved in oral tolerance, i.e., interleukin (IL)-4, IL-10 and transforming growth factor-β, were higher in OVA- than in PBS-fed C rats, but not in ZD rats. Apoptosis was higher in OVA- than in PBS-fed C rats but not different between OVA- and PBS-fed ZD rats. Inflammation and ulcerations that were not present in ZD rats on d 7 (ZD7) developed in OVA- or PBS-fed ZD rats. Compared with ZD7 rats, tumor necrosis factor-α and cytokine-induced neutrophil chemoattractant were higher in OVA- and PBS-fed ZD rats, whereas interferon-γ increased only in OVA-fed ZD rats. In conclusion, zinc deficiency suppresses oral tolerance through dysregulation of cytokine expression and lack of antigen-specific clonal deletion. We suggest that abrogation of tolerance may lead to development of mucosal inflammation and damage. J. Nutr. 133: 191–198, 2003.

KEY WORDS: • zinc deficiency • oral tolerance • intestine • cytokines • rats

The intestinal mucosa is an enormous surface that is continuously exposed to a myriad of intraluminal antigens. The immune system of the gut has to be able to protect the mucosa against pathogens but also must avoid hypersensitivity reactions to food proteins, normal bacterial flora and other environmental macromolecules. Oral tolerance is a specific suppression of cellular and humoral cell-mediated immune responses to orally administered antigen upon subsequent immunization with the same antigen to prevent immune reactions to dietary antigens (1–6). There is a large body of evidence that adverse reactions to foods have an immunological basis and may represent a suppression of tolerance to components of gut flora and food (2,7). Breakdown of oral tolerance may lead to the development of mucosal immunopathology directed against environmental antigens or autoantigens and thus to autoimmune diseases. Indeed, oral tolerance has been employed successfully for treatment of human autoimmune diseases (1) and to suppress experimental autoimmune myasthenia gravis (8), uveitis (9), rheumatoid arthritis (10), autoimmune encephalomyelitis (11) and colitis (12).

Tolerance may occur by a number of mechanisms depending on the dose and nature of the antigen orally administered. The induction of clonal deletion of antigen-specific T lymphocytes and anergy of T cells have been demonstrated to occur after high doses of oral antigen (13,14). Feeding a low antigen dose is generally associated with active suppression (1,15). This is generated by food antigen uptake and processing in Peyer's patches and villous epithelium, which induce the development of T-helper (Th)4 cells secreting immunosuppressive cytokines such as interleukin (IL)-4 and IL-10, as well the development of Th3 lymphocytes producing transforming growth factor (TGF)-β (16). These cytokines can suppress immune reactions and antagonize the expression of Th1 proinflammatory cytokines. The Th2 cells can emigrate to peripheral sites where they prevent continuing activation of pathogenic Th1 cells. Although it is generally believed that active suppression and apoptosis of T-cells act separately in induction of tolerance, there is also evidence that both these mechanisms may be present at the same time in animals in which tolerance was induced (17,18).

In addition to the dose or nature of antigen, it has been suggested that altered immunological status, increased inflammation, dysfunction or damage in the epithelial barrier and

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imbalance in nutritional status may influence the immunologic outcome after oral antigen administration and lead to a suppression of oral tolerance (19–21). Apart from recent studies showing that patients with inflammatory bowel disease failed to elicit oral tolerance (22), data concerning the effect of these variables on oral tolerance are still lacking.

Zinc deficiency may affect the immune system by causing atrophy of lymphoid tissue, decreasing antibody production, reducing T cell proliferation and cytokine production in response to mitogens and specific antigens, and inducing an imbalance between Th1 and Th2 functions (23–26). Zinc deficiency may also affect several functions of different tissues, and the intestine is one of the most sensitive tissues to this nutritional deficiency (27). In previous studies, we have shown that the intestine of zinc-deficient rats developed inflammation and ulcerations, with increasing severity between 20 and 40 d of zinc deficiency (28,29). We have also reported that these alterations were associated with increased expression of proinflammatory cytokines (29). Considering the different effects of zinc deficiency, we hypothesized that zinc deficiency may affect oral tolerance induction.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River, Como, Italy) weighing 200–220 g were housed in stainless steel cages and maintained at 23°C with a 12-h light/dark cycle. They were randomly assigned to three groups of rats: one group of zinc deficient (ZD) rats was fed a ZD diet (<2 mg Zn/kg), one group of control (C) rats was fed the ZD diet supplemented with zinc carbonate to 50 mg Zn/kg, and one group of pair-fed (PF) rats was fed the C diet according to the mean intake of the ZD rats, as a control for the inanition associated with zinc deficiency. All rats were fed the diets for 28 d with the exception of some ZD rats that were killed on d 7 (ZD7), n = 8) before the induction of tolerance, which is described below. The rats had free access to food. The water was deionized and distilled. Final body weight and daily food intake were recorded. The composition of the ZD diet was as in a previous study (28), but contained hydrolyzed meat protein instead of egg white. The diets were purchased from Italy.

Induction of oral tolerance and immunization. After 7 d of dietary treatment, each group of rats (n = 120 for C and ZD and 24 for PF rats) was divided in two subgroups that were fed either ovalbumin (OVA; grade V; Sigma, Milan, Italy) to induce tolerance, or PBS. The subgroups were called OVA-fed and PBS-fed. OVA was given as a low dose (<7 mg) dissolved in deionized water, by gavage, 3 times/wk. PBS was given for the same period of time. The oral dose of OVA was chosen on the basis of previous results (18,30,31). On d 14, all rats were immunized by injection of 300 mg/L OVA emulsified in 96-well plates (Costar, Rome, Italy) at 37°C in a humidified atmosphere with 5% CO2. Cell viability was measured by trypan blue exclusion and was similar in all groups.

Because zinc deficiency may affect the immune response, we first determined whether zinc deficiency reduced the capacity of lymphocytes to proliferate by measuring the proliferative response to a mitogen. Cells were treated with 2.5 μg/mL of concanavalin A (ConA; Sigma) for 72 h and pulse-labeled with 1.17 KBq of [3H]thyridine (24–48 h) and then subjected to 35 cycles of PCR, followed by a last cycle at 72°C for 7 min on a DNA thermal cycler (Applied Biosystems, Rome, Italy). The PCR cycle conditions were: 30 s at 95°C and 2.5 min at 62°C. The relative intensity of the bands was analyzed by Scion image software (Scion, Frederick, MD). The ratio of cytokine/
GAPDH mRNA intensities was used to evaluate the relative levels of expression.

**Cytokine secretion.** Culture supernatants from unstimulated and OVA-stimulated (5 g/L) MLN and spleen cells were tested for the presence of IL-4 and IL-10 after different times of culture (72 h for IL-4 or 120 h for IL-10), using rat IL-4 and IL-10 ELISA kits (Endogen, Milano, Italy). For IL-4, supernatants were concentrated 10 times.

**Apoptosis.** MLN and spleen cells were cultured in the presence of 5 g/L of OVA for 96 h. Cells were fixed in 40 g/L paraformaldehyde (Sigma), pH 7.4, in PBS. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay using a “In situ cell death detection, POD” kit (Boheringer, Milano, Italy), under a light microscope (Leitz, Wetzlar, Germany).

**Histologic analysis.** Pieces of jejunum (3 cm) of NZD and of OVA- or PBS-fed NZD rats were dissected, fixed in Bouin’s solution for 12 h and embedded in paraffin at 58°C. Sections (7 μm) were stained with Mallory stain and then examined by light microscopy. We decided to undertake a histological evaluation of the jejunum on the basis of our previous results indicating that this region was affected the most by zinc deficiency (28).

**Myeloperoxidase (MPO) activity.** MPO activity was measured in the intestine of NZD, and of OVA- or PBS-fed NZD rats as previously described (27). The MPO activity was expressed per micromgram of protein. Protein concentration in the assay was determined using a Bio-Rad protein assay kit (Bio-Rad, Milano, Italy).

**Zinc in diets and serum.** Zinc concentration in diets and serum was determined by flame atomic absorption spectrophotometry using a SpectraAA-400 model atomic absorption spectrometer (Varian, Melbourne, Australia).

**Statistical analysis.** Data were evaluated by two-way ANOVA followed by Tukey’s test, except data of cytokine expressions (Fig. 3), which were analyzed by three-way and one-way ANOVA followed by Fisher’s test, and data of NZD group (Tables 1 and 3 and Fig. 5), which were compared with the NZD groups by Student’s test. Variance homogeneity and normal distribution were verified with Bartlett’s test and the Shapiro-Wilk test, respectively. Correlation analysis was performed using the Pearson’s test. Differences with *P* values < 0.05 were considered significant.

## RESULTS

### Body weight, food intake and serum concentration.

Within the C, NZD and PF groups, the OVA- and PBS-fed subgroups did not differ in body weight, food consumption or serum zinc concentration (Table 1). On the other hand, these variables were significantly lower in NZD rats than in C rats.

The PF rats had body weights and serum zinc levels higher than those of NZD rats but lower than those of C rats. In NZD rats, food intake was lower and serum zinc higher than in NZD rats.

**Proliferative response to ConA.** After ConA stimulation, the proliferative response of MLN and spleen cells cultured in medium containing FCS was strong and not different between OVA- and PBS-fed C and NZD rats. The stimulation index (cpm of stimulated/cpm of unstimulated cells) ranged from 97 ± 12 to 135 ± 19 in MLN and from 125 ± 14 to 148 ± 19 in spleen. The cell proliferation of NZD rats was lower than that of C rats when the culture medium contained serum of the NZD rats instead of FCS (data not shown). These results are in agreement with our previous reports that 30 d of zinc deficiency did not affect lymphocyte proliferation when lymphocytes were cultured in medium containing FCS (32).

**Proliferative response to in vitro OVA stimulation.** The lower responsiveness of OVA-fed C rats to in vitro OVA stimulation compared with that of PBS-fed C rats indicated that oral tolerance was induced in C rats (Fig. 1). The strong proliferative response of MLN and spleen cells from OVA-fed NZD rats to in vitro OVA stimulation was not different from that of PBS-fed NZD and C rats, indicating that oral tolerance was not elicited in NZD rats. The cell proliferation after OVA stimulation of OVA- or PBS-fed PF groups did not differ from that of OVA- or PBS-fed C rats, respectively, indicating that zinc restriction did not affect oral tolerance induction and that the loss of oral tolerance in NZD rats was due to zinc deficiency and not to food restriction. All of these experiments were conducted in FCS-containing medium. When the effect of culture medium containing serum of the NZD rats was tested in preliminary experiments, the results indicated that the OVA proliferative response of lymphocytes from OVA- or PBS-fed NZD rats was lower with NZD serum than with FCS (data not shown). However, also with NZD serum, the proliferation of OVA-fed NZD rats was higher than that of OVA-fed C rats and not different from that of PBS-fed NZD rats, indicating that the high OVA responsiveness of OVA-fed NZD rats was not due to the zinc in FCS but rather, the abrogation of oral tolerance. Thus, we performed the experiments with medium containing FCS to maintain the cells in the best conditions.

**DTH response.** In C rats, a strong DTH response was elicited only in those fed PBS, whereas in NZD rats, both OVA- and PBS-fed rats showed marked DTH responses that did not differ from that of PBS-fed C rats (Fig. 2). It has been reported that zinc deficiency may affect the DTH response. In this study, the elevation of DTH in both OVA- and PBS-fed NZD rats could be due to our experimental model using OVA as the

### Table 1

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<thead>
<tr>
<th></th>
<th>C</th>
<th>ZD</th>
<th>OVA-fed</th>
<th>PBS-fed</th>
<th>OVA-fed</th>
<th>PBS-fed</th>
<th>OVA-fed</th>
<th>PBS-fed</th>
<th>ZD</th>
<th>Main effect&lt;sup&gt;4&lt;/sup&gt;</th>
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<tr>
<td><strong>Final body weight, g</strong></td>
<td>410 ± 40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>415 ± 41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>175 ± 21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>181 ± 23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>268 ± 27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>271 ± 29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>191 ± 17</td>
<td></td>
<td></td>
<td>*P &lt; 0.001</td>
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<tr>
<td><strong>Food consumption, g</strong></td>
<td>461 ± 50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>468 ± 50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>242 ± 28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>247 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>242 ± 28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>247 ± 25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66 ± 8&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td><strong>Serum zinc, μg/mL</strong></td>
<td>18.6 ± 1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.9 ± 1.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.3 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.1 ± 0.43&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.5 ± 1.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.7 ± 0.98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.2 ± 1.92*</td>
<td>*P &lt; 0.001</td>
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</table>

<sup>1</sup> Data are the means ± SD of at least 5 for NZD<sup>+</sup> group or 12 rats for all the other groups.

<sup>2</sup> Food consumption was measured for 7 d for NZD<sup>+</sup> group or 28 d for all the other groups.

<sup>3</sup> C, D and PF means in a row without a common letter differ (*P < 0.001). * Different from OVA- and PBS-fed NZD groups, *P < 0.001.

<sup>4</sup> Significant effect in the two-way ANOVA. Treatment and the diet × treatment interaction were not significant.
In addition, cytokine expressions of OVA-fed ZD rats after OVA stimulation were significantly lower than those of OVA-fed C rats (Fig. 3).

The amounts of IL-4 and IL-10 secreted by MLN and spleen cells from C rats were significantly lower in PBS- than in OVA-fed rats (Fig. 4). In MLN cells from ZD rats, the IL-4 secretion of OVA-fed did not differ from that of PBS-fed rats, and the IL-10 secretion was higher in PBS- than in OVA-fed rats. In spleen cells from ZD rats, the production of both IL-4 and IL-10 was not different between OVA- and PBS-fed rats. Moreover, the IL-4 and IL-10 secretions of OVA-fed ZD rats of both MLN and spleen were lower than those of OVA-fed C rats.

We examined the statistical analyses of linear and multiple regressions and noted the dependence of the proliferative response to OVA on cytokine expression and serum zinc level. Indeed, the linear regression used to correlate the MLN proliferative response to OVA with cytokine expressions high-

antigen in contrast to other studies in which a pathogenic or toxigenic antigen was used (33–35).

Expression of cytokines primarily involved in oral tolerance. The gene expressions of IL-4, IL-10 and TGF-β of MLN and spleen lymphocytes after in vitro OVA stimulation were significantly higher in OVA- than in PBS-fed C rats (Fig. 3). On the other hand, in MLN lymphocytes from ZD rats, IL-4 of OVA-fed was not different from that of PBS-fed rats, IL-10 was higher in PBS- than in OVA-fed rats and TGF-β was very low in both OVA- and PBS-fed rats. In stimulated spleen cells from ZD rats, the expressions of these cytokines in both OVA- and PBS-fed rats did not differ from one another.

FIGURE 1 Proliferative response of mesenteric lymph node (MLN) and spleen cells of ovalbumin (OVA)- or PBS-fed control (C) and zinc-deficient (ZD) rats to in vitro OVA stimulation. The results are expressed as stimulation index (cpm of stimulated/cpm of unstimulated cells). Data are the means ± SD of at least 20 rats. Means without a common letter differ (P < 0.01). Diet and treatment affected the proliferative response (both P < 0.001) and there was a diet x treatment interaction (P < 0.001).

FIGURE 2 Effect of oral administration of ovalbumin (OVA) on the delayed-type hypersensitivity (DTH) response of OVA- or PBS-fed control (C) and zinc-deficient (ZD) rats. The results are expressed as difference between the left and right foot pad. Data are the means ± SD of at least 10 rats. Means without a common letter differ (P < 0.05). Diet and treatment affected the proliferative response (both P < 0.001) and there was a diet x treatment interaction (P < 0.001).

FIGURE 3 Gene expression of cytokines primarily involved in oral tolerance induction of mesenteric lymph node (MLN) and spleen cells of ovalbumin (OVA)- or PBS-fed control (C) and zinc-deficient (ZD) rats after in vitro OVA stimulation, analyzed by RT-PCR. The figure represents the densitometric values of IL-4, IL-10 and TGF-β mRNA normalized to GAPDH mRNA. Data are the means ± SD of at least 6 separate experiments. In each experiment, 4 to 6 rats were pooled. Means without a common letter differ (P < 0.05). Because the three-way ANOVA indicated a significant interaction of the 3 main effects for all of the cytokines (P < 0.001), the individual treatment groups were analyzed by one-way ANOVA.

FIGURE 4 Expression of cytokines primarily involved in oral tolerance induction of mesenteric lymph node (MLN) and spleen lymphocytes after in vitro OVA stimulation were signifi-

cantly higher in OVA- than in PBS-fed C rats (Fig. 3). On the other hand, in MLN lymphocytes from ZD rats, IL-4 of OVA-fed was not different from that of PBS-fed rats, IL-10 was higher in PBS- than in OVA-fed rats and TGF-

/β was very low in both OVA- and PBS-fed rats. In stimulated spleen cells from ZD rats, the expressions of these cytokines in both OVA- and PBS-fed rats did not differ from one another.
lighted a high correlation coefficient ($R^2$) for IL-4, IL-10 and TGF-$\beta$ between OVA- and PBS-fed C rats, indicating that each of these cytokines explains 95–96% of the variance in MLN proliferative response to OVA antigen, whereas a low $R^2$ was found between OVA-fed and PBS-fed ZD rats (Table 2). When the cytokine expressions were correlated with the serum zinc concentrations, a high $R^2$ was found for each of these cytokines between OVA-fed C and ZD rats, indicating that IL-4 accounts for 88% and IL-10 and TGF-$\beta$ for 96% of the variance in serum zinc level (Table 2). The $R^2$ was low when groups with similar zinc concentrations were compared, i.e., between OVA- and PBS-fed C and ZD rats (data not shown). When the proliferative response to OVA was correlated with the serum zinc concentrations, the $R^2$ was 0.86 between OVA-fed C and ZD rats and 0.72 between PBS-fed C and ZD rats. The $R^2$ was low when groups with similar zinc level. The multiple regression analysis of proliferative response to OVA vs. the three cytokines yielded an $R^2$ of 0.9710 ($P < 0.001$) between OVA- and PBS-fed C rats and an $R^2$ of 0.607 ($P = 0.11$) between OVA- and PBS-fed ZD rats, confirming that in C rats, all of the cytokines are involved in controlling proliferation, whereas this is not the case in ZD rats.

**Expression of proinflammatory cytokines in intestinal mucosa.** Compared with ZD$_7$ rats, the constitutive levels of TNF-$\alpha$ and CINC were significantly greater in both OVA- or PBS-fed ZD rats, whereas the expression of IFN-$\gamma$ was significantly higher in OVA-fed but not in PBS-fed ZD rats (Fig. 5).

**Assessment of apoptosis.** OVA-fed C rats had significantly more apoptotic cells in MLN and spleen cells after in vitro OVA stimulation than PBS-fed C rats (Fig. 6). The percentage of apoptotic cells in both OVA- or PBS-fed ZD rats did not differ from one another or from OVA-fed C rats.

**Morphology and inflammatory status of gut mucosa.** The morphological analysis did not show any alterations in jejunum of ZD$_7$ rats, whereas several ulcerations, marked edema and inflammatory cell infiltration were present in OVA-fed ZD rats (Fig. 7). The mucus of PBS-fed ZD rats was similar to OVA-fed ZD rats (not shown).

The intestinal MPO activity, a marker of macrophage and neutrophil infiltration, did not differ between OVA- and PBS-fed ZD rats and was significantly greater than that of OVA- or PBS-fed C rats (Table 3). The MPO activity of ZD$_7$ rats was significantly lower than that of OVA- or PBS-fed ZD rats.

### TABLE 2

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<th>Slope</th>
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<td>Proliferative response to OVA and cytokine expression</td>
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<td>OVA-fed C + PBS-fed C1</td>
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<tr>
<td>IL-4</td>
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<td>0.96</td>
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<tr>
<td>TGF-$\beta$</td>
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<td>0.96</td>
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<td>TGF-$\beta$</td>
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<td>0.3</td>
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<td>Cytokine expression and serum zinc concentration</td>
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<tr>
<td>IL-4</td>
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<td>IL-10</td>
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<td>TGF-$\beta$</td>
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<td>IL-10</td>
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<td>TGF-$\beta$</td>
<td>0.92</td>
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1 Rats were grouped by diet.
2 Rats were grouped by treatment (OVA and PBS).
DISCUSSION

Recent studies have suggested that oral tolerance is mediated by several mechanisms and that different variables may play a role in this phenomenon. Nutritional deficiencies, dysfunction in the epithelial barrier, increased permeability to luminal antigens, increased intestinal inflammation and infections may affect the development of oral tolerance. We showed recently that zinc deficiency induced ulcerations, inflammation and increased expression of proinflammatory cytokines in rat intestine. Here, we report that zinc deficiency may affect the induction of oral tolerance. In this study, the ZD rats were orally fed OVA after 7 d of zinc deficiency, that is, in the early stages of zinc deficiency. Because it has been demonstrated that systemic tolerance develops soon after antigen feeding (3), and activation of cytokine production in gut-associated lymphoid tissue occurs within a few hours of antigen feeding (30), our findings indicate that only a few days of zinc deficiency are sufficient to suppress tolerance.

A primary mechanism of oral tolerance is active suppression via generation of regulatory cells (Th2 and Th3) which, upon subsequent recognition of antigen, secrete down-regulatory cytokines. Some investigators have shown the importance of IL-4 and IL-10 in the development of oral tolerance using mice genetically engineered to lack one or both of these cytokines (36). IL-4 is also a differentiation factor for TGF-β-secreting Th3 cells (37). TGF-β is a potent immunosuppressive and anti-inflammatory cytokine; it is essential for the induction of oral tolerance (38,39). In agreement with these findings, our data showed an up-regulation of IL-4, IL-10 and TGF-β expressions of both MLN and spleen cells from OVA-fed compared with PBS-fed C rats. On the other hand, a dysregulation in the expression of these cytokines was associated with the lack of oral tolerance in ZD rats. The strong association of proliferative response to OVA with IL-4, IL-10 and TGF-β expressions as well the association of these cytokines with serum zinc in C rats but not in ZD rats, is a further indication that the suppression of oral tolerance in ZD rats is mediated by cytokine dysregulation and that the induction of tolerance is dependent on serum zinc level.

Our data indicate a suppression of oral tolerance at intestinal and peripheral sites in zinc deficiency. Previous studies have demonstrated a role of the thymus in oral tolerance induction by generating regulatory cells that can transfer tol-

![FIGURE 6](https://academic.oup.com/jn/article-abstract/133/1/191/4687649)

**FIGURE 6** Induction of apoptosis in mesenteric lymph node (MLN) and spleen cells of ovalbumin (OVA)- or PBS-fed control (C) and zinc deficient (ZD) rats after in vitro OVA stimulation. The results are expressed as the percentage of apoptotic cells. Data are the means ± SD of at least 6 experiments. Means without a common letter differ (P < 0.05). Diet and treatment affected the proliferative response (both P < 0.05) and there was a diet × treatment interaction (P < 0.01).

![FIGURE 7](https://academic.oup.com/jn/article-abstract/133/1/191/4687649)

**FIGURE 7** Jejunum of a d-7 zinc-deficient (ZD7) (A) and an ovalbumin (OVA)-fed ZD rat (B). Before OVA feeding (d 7; ZD7) no alterations were present, whereas several ulcerations, marked edema and inflammatory cell infiltration were evident in OVA-fed rats. The arrowhead and arrow indicate one of the sites of edema and ulcerations, respectively.
Because zinc deficiency may affect thymus function and T cell subsets (23,26), it is possible that a suppression of oral tolerance also occurred in the thymus in zinc deficiency.

In this study, a low dose of OVA was used to induce tolerance. A low dose of antigen induces active suppression (1,15), whereas a high antigen dose induces clonal deletion (14). However, it has also been demonstrated that the two mechanisms may be present at the same time (17,18). In agreement with these findings, our results of increased apoptosis of OVA-specific T cells in OVA-fed C rats suggest that both active suppression and clonal deletion are associated with tolerance induction. A high percentage of apoptotic cells was also present in ZD rats after in vitro OVA stimulation, but percentages did not differ between OVA- and PBS-fed groups, suggesting that the apoptotic cells were not OVA-specific T cells. Indeed, our preliminary experiments have shown that Fas expression, one of the principal mediators of antigen-specific deletion in oral tolerance (41,42), was expressed at a high level in OVA-fed C rats but at a very low level in OVA-fed ZD rats (data not shown). Further experiments are necessary to explain these results. Thus, the high level of cell death in ZD rats could be caused by zinc deficiency per se, in agreement with the results of previous studies indicating that zinc deficiency can induce apoptosis (43–45).

The consequences of a breakdown in the state of oral tolerance may be dramatic. In fact, tolerance is necessary to prevent the development of an immune response to innocuous dietary antigens as well to bacterial antigens normally present in the gastrointestinal tract. Thus, the abrogation of tolerance may result in mucosal immunopathology and enhanced reactivity to autoantigens that may lead to the development of autoimmune diseases. The results here reported of the intestinal histological analysis, MPO activity and proinflammatory cytokine expressions of ZD rats before OVA feeding did not show any of the inflammation and cell damage that were present at the end of the experimental period. The expression of IFN-γ indicates that the inflammatory status was more established in OVA- than in PBS-fed ZD rats. Thus, although zinc deficiency may cause intestinal damage through mechanisms other than suppression of oral tolerance, our data suggest that the suppression of oral tolerance may have generated an inflammatory status with a progression of inflammation and mucosal damage throughout the experimental period.

In conclusion, our results indicate that a moderate zinc deficiency is sufficient to suppress oral tolerance by mechanisms involving dysregulation of cytokines primarily responsible for oral tolerance and lack of antigen-specific clonal deletion. In addition, our findings provide further support that suppression of oral tolerance may lead to gut inflammation and damage.

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LITERATURE CITED