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# Ethanol Affects the Absorption and Tissue Distribution of Orally Administered Antigens in Mice

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## ABSTRACT

*The aim of this work was to evaluate the effects of ethanol on the adsorption and tissue distribution of orally administered antigens in mice. Results showed that ethanol reduced the level of anti-ovalbumin IgA antibodies in intestinal fluid for the mice treated orally with a palmitoyl–ovalbumin conjugate. Ethanol was administered intragastrically to mice at 5 g/kg body weight for 14 days (chronic treatment), or 10 g/kg body weight every 7$^{th}$ day up to 14 days (acute treatment). Thereafter, $^{99m}$technetium-labeled antigens were administered and lymphoid tissues were collected. Ethanol interfered with the transport of ovalbumin to the liver. Moreover, the transport of palmitoyl–ovalbumin to mesenteric lymph nodes was reduced 6 h after the antigen administration. In conclusion, there was a relationship between the suppression of ethanol-mediated specific local IgA responses and the decreased transport of palmitoyl–ovalbumin to mesenteric lymph nodes.*

**Key words:** ethanol, absorption, biodistribution, technetium, immune response

## INTRODUCTION

Orally administered antigens interact with gut-associated lymphoid tissue, which comprises of epithelial cells and intra-epithelial and lamina propria lymphocytes (Brandtzaeg 1985; McGhee et al. 1992). These antigens are drained into Peyer's patches and mesenteric lymph nodes (MLNs), or the liver. The most common consequence of the oral administration of antigens is the oral tolerance induction that comprises a state of systemic humoral and cellular responses suppression to fed antigens (Challacombe and Tomasi 1980; Titus and Chiller 1981). Moreover, antigens can activate the intestinal mucosal immune system, resulting in antigen-specific IgA production, or systemic immunization.

Several mechanisms that induce the suppression of antigen-specific immune responses in oral tolerance have been proposed, including clonal deletion, anergy, and dominant tolerance involving active immune suppression by CD4+ CD25+ Foxp3+ regulatory T cells (Tregs). This last mechanism is of particular relevance to the present study because it involves self-reactive, thymically derived, naturally occurring Tregs and inducible Tregs produced from the antigen-specific naïve CD4+ CD25− Foxp3− T cells in peripheral areas under certain environmental conditions (Hori et al. 2003; Keir et al. 2008; Curotto de Laifaille and Lafaille 2009; Zhou et al. 2009).

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Thus, several factors such as the route of antigen uptake determine the type and magnitude of the ensuing immune response against orally administered antigens (Emark et al. 1995; Matsunaga et al. 2001; Jonh et al. 2002). Antigens are taken up by either the M cells in Peyer's patches, or epithelial cells in the villi, and this uptake is dependent largely on the nature (i.e., particulate or soluble) and size of the antigen (Nakaoka et al. 1996; Tabata et al. 1996).

Factors that affect the integrity of the intestinal wall can alter the antigen absorption and tissue distribution, and consequently, immunological responses. For instance, ethanol can alter the morphology and physiology of the intestinal wall, promote serious damage to the gastric mucosa by destroying 10–50% of villi and enhance intestinal permeability (Andrade et al. 2006; Beck et al. 1996). A previous study had shown that palmitoyl–ovalbumin (palmitoyl–ova), not the naïve ova, was efficient in generating the humoral and cellular immunity (Oliveira et al. 2002). Palmitoyl–ova also stimulated the production of IgA antibodies (Oliveira et al. 2002). By contrast, the levels of IgA antibodies were lower in the intestinal fluid from ethanol-treated mice (Gusmão et al. 2004); however, systemic immunization and oral tolerance against palmitoyl–ova and ova, respectively, were not affected by ethanol (Gusmão et al. 2004). This study evaluated the effects of ethanol on the absorption and tissue distribution of ova and palmitoyl–ova after oral administration.

## MATERIALS AND METHODS

### Animals

Male and female B6D2F1 mice (age, 7–9 weeks; n = 12 per group), alternatively known as (C57Bl/6xDBA/2) F1 hybrids, were obtained from the breeding unit at the Federal University of Minas Gerais (UFMG – Belo Horizonte, Brazil). Studies were conducted in accordance with the Brazilian Society for Neuroscience and Behaviour Guidelines for Animal Experimentation.

### Antigens

Chicken egg albumin (ova, grade V) was purchased from the Sigma Chemicals Co. (St. Louis, MO, USA). Lipid–ova conjugates (i.e., palmitoyl–ova) were prepared as described previously (Oliveira et al. 1998). In brief, 1.0 g of the N-hydroxysuccinamide ester of palmitate (Sigma) was dissolved in 100 mL of absolute ethanol at 55°C. This mixture was then combined with 800 mL of phosphate-buffered saline containing 4.0 g ova and 0.6% deoxycholate (wt/vol, Sigma) at 37°C. After overnight incubation at room temperature, the mixture was centrifuged and the precipitate was collected. The precipitate was washed with 50 mM $NaHCO_3$, and a suspension was prepared. After freezing the suspension in the liquid nitrogen, the conjugate was lyophilized.

### Ethanol treatment

Ethanol was administered intragastrically to mice at 5 g/kg body weight for 14 days (chronic treatment), or 10 g/kg body weight every 7$^{th}$ day up to 14 days (acute treatment).

### Labeling antigens with technetium ($^{99m}$Tc)

Ova and palmitoyl–ova were incubated with 10 µl $SnCl_2$ (2 mg/ml $SnCl_2$ in 0.25 M HCl), 10 µl $NaBH_4$ (2 mg/ml $NaBH_4$ in 0.1 M NaOH), and 4 MBq of $^{99m}$Tc for 20 min at room temperature. Labeling efficiency, determined as described previously (Araújo et al. 2002), was 95% ± 0.5 (n = 50). The antigenic integrity of $^{99m}$Tc-labeled antigens was assessed by affinity chromatography and labeling did not affect the antigenic properties. The mixture was concentrated using a disposable microconcentrator to remove the unbound $^{99m}$Tc before use (Alves et al. 2005).

**Table 1 –** Ethanol treatment protocol.

| Ethanol treatment protocol | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Day** | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Acute ethanol treatment g/kg body weight | 10 | - | - | - | - | - | - | 10 | - | - | - | - | - | - | 10 |
| Chronic ethanol treatment g/kg body weight | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

### Biodistribution of labeled antigens

Aliquots containing 20 mg of labeled antigens (20-mg aliquots) were administered by gavage to mice. The mice were sacrificed 1 and 6 h after antigen administration, bled, and internal organs such as the stomach, MLNs, spleen, liver, small and large

intestines, and Peyer's patches were collected. The organs were washed briefly with saline, dried, and weighed. The small intestine was divided into three segments of equal length. Intestinal fluid was obtained after rinsing each segment with 1.0 ml saline. Peyer's patches from each segment were removed and tested. Radioactivity was measured in different organs using an automatic scintillation apparatus (ANSR-Abbot, USA). To determine the rate of radioactive decay and to standardize the assay, the number of counts per minute was measured in the aliquots.

**Statistical analysis**

Differences between the ova (n = 12) and palmitoyl–ova (n = 12) groups were evaluated by two-tailed unpaired Student's *t*-test. Statistics were performed using GraphPad Prism 5 software for Windows (San Diego, CA, USA). $p < 0.05$ was considered statistically significant. Data are from at least three experiments, and results are expressed as mean ± S.D.

## RESULTS AND DISCUSSION

The acute and chronic administration of ethanol caused macro- and microscopic morphological changes in the intestine, which caused a reduction in villus height (data not shown).

To determine the effects of morphological changes induced by ethanol on the absorption and tissue distribution of orally administered antigens (ova and palmitoyl-ova), the presence of $^{99m}$Tc-labeled antigens in Peyer's patches, MLNs, liver, spleen, and blood was analysed. Figure 2 shows the distribution of labeled antigens 1 h after administration. An acute dose of ethanol resulted in a significant increase in the level of palmitoyl–ova in Peyer's patches 1 h after antigen administration. In addition, the level of $^{99m}$Tc-labeled palmitoyl–ova increased in the liver of ethanol-treated mice; however, the level of $^{99m}$Tc-labeled ova decreased in this organ. Ethanol did not affect antigen distribution in MLNs and blood.

Figure 2 also shows the absorption and tissue distribution of labeled antigens 6 h after administration. The levels of palmitoyl–ova increased in the spleen and blood. By contrast, ethanol reduced the level of palmitoyl–ova in MLNs. The absorption and distribution of $^{99m}$Tc-labeled ova in untreated and ethanol-treated mice were comparable 1 and 6 h after, except that a lower level was observed in the liver after 1 h. The absorption and distribution of $^{99m}$Tc-labeled ova and palmitoyl-ova were similar in the mice treated with acute or chronic doses of ethanol (data not shown).

Mucosal tissues are constituted by physiological and immunological barriers that restrict the entry of the antigen into organisms. Even though the glycocalyx, mucins, secretory IgA antibodies, and junctional structures reduce the macromolecule intestinal transport, significant amounts of soluble and particulate antigens penetrate in lamina propria through paracellular pathway, enterocytes or M cells (Sun et al. 1997; Lennernãs 1998). The cellular events that modulate antigen uptake and processing appear to determine whether feeding of antigen induces the tolerance, or primes an immune response in the gastrointestinal tract (Mestecky et al. 1997; Gamvrellis et al. 2004).

Ethanol may also play a role in the uptake of antigens in the gut and in the immune responses triggered by orally administered antigens. An earlier study has demonstrated a decrease in the level of IgA antibodies against palmitoyl–ova in the intestinal fluid from ethanol-treated mice; however, systemic immunological events induced by orally administered antigens were not affected (Gusmão et al. 2004). In this study, $^{99m}$Tc, a radionuclide widely used to label the drugs, peptides and antibodies was used for biodistribution studies (Jamar et al. 2002). Free $^{99m}$Tc accumulates largely in the thyroid and stomach wall (Argonne National Laboratory 2005).

The paracellular pathway is restricted to the transport of ions and anions. However, pathologies, or ethanol consumption increase the permeability of the intestinal wall due to the junctional barriers damage (Borrielo, 2001). The higher antigen levels observed in the liver and blood at 1 and 6 h, respectively, illustrated an increase in the permeability to palmitoyl–ova in ethanol-treated mice. On the other hand, ethanol did not affect the absorption of ova and only the ova level in the liver was reduced at 1 h. These showed that the antigen absorption in ethanol-treated mice was dependent on the nature of the molecule. In other words, the absorption of hydrophobic and particulate antigens was higher than that of soluble antigens.

The transport of macromolecules via enterocytes, or M cells was also influenced by their nature and size. For instance, soluble molecules are

transported via enterocytes with a similar kinetics (Mayer et al. 1999). On the other hand, the transport of particulate antigens via enterocytes is limited because of their size; therefore, these antigens are mainly transported by M cells (Eldrige et al. 1991; Desai et al. 1996; Chen and Langer 1998; Matsunaga et al. 2001). In agreement with the previous observations, the uptake of palmitoyl–ova, a particulate and hydrophobic antigen, by Peyer's patches was higher than that of ova (Oliveira et al. 2002; Oliveira et al. 2007). However the uptake of palmitoyl-ova in ethanol-treated mice was higher after 1 h, but not after 6h after antigen administration. In other words, ethanol reduced the maintenance of palmitoyl-ova in the Peyer´s patches.

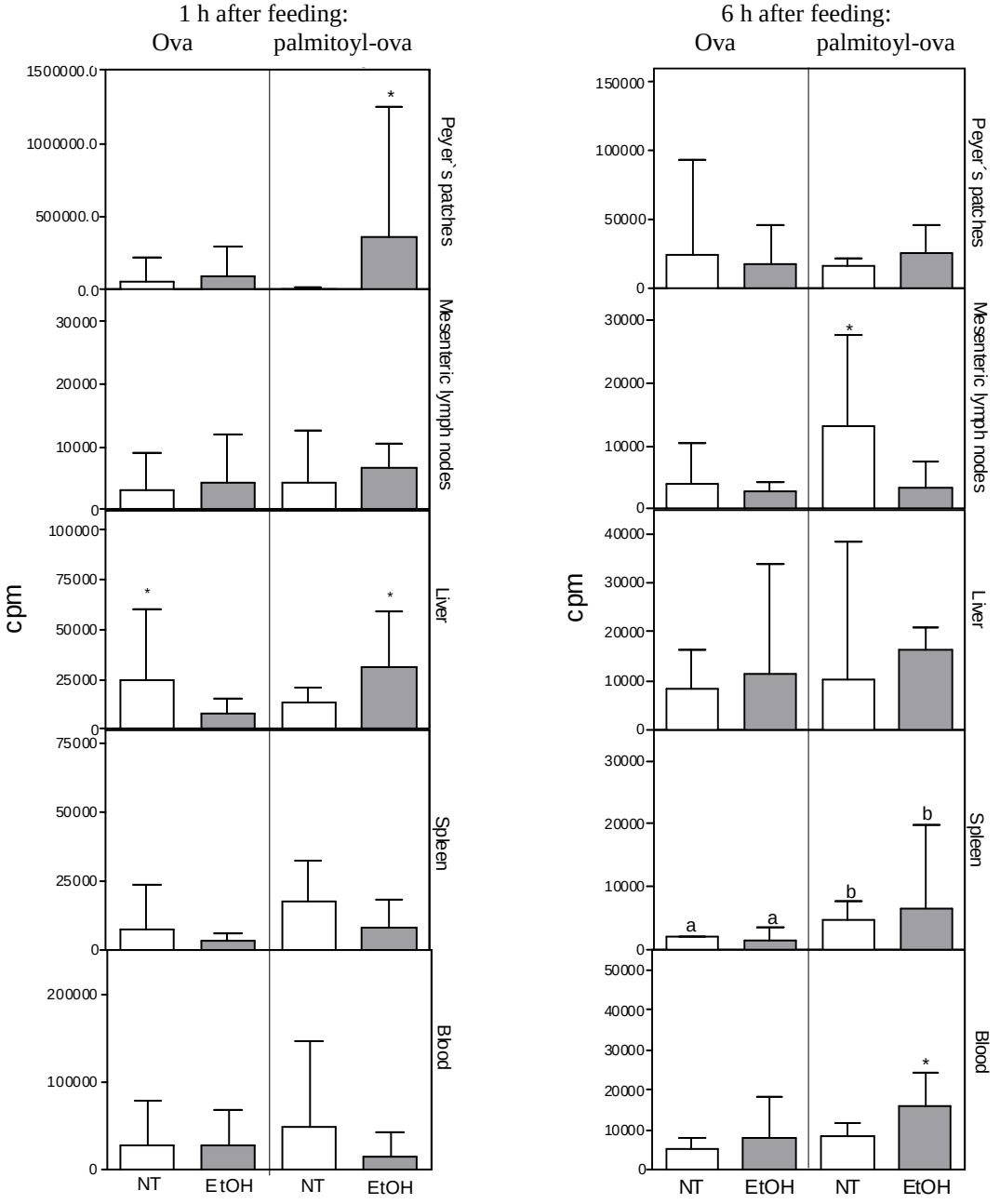


**Figure 2 -** Uptake of radiolabeled antigens in Peyer's patches, mesenteric lymph nodes, liver, spleen and blood. B6D2F1 mice received acute doses of ethanol (EtOH; n = 12) or saline (NT; n = 12). Treated and untreated animals were fed $^{99m}$Tc-labeled ova or $^{99m}$Tc-labeled palmitoyl–ova, and organs were removed and weighed. $p$ (a/b) < 0.05 or $p$ (*) < 0.05 represents a significant difference between groups.

The most significant effect of ethanol administration on palmitoyl–ova distribution was a reduction in antigen levels in MLNs. Hydrophobic antigens such as palmitoyl–ova are drained by MLNs and subsequently delivered into the bloodstream (Reddy and Murthy 2002). Within MLNs, immune responses are triggered by the activation and differentiation of resident cells. In contrast to contradictory reports on the importance of Peyer's patches, MLNs play a crucial role in the induction of mucosal immunity and tolerance. For instance, studies have shown an inhibition of mucosal immunization induced by the particulate antigens in the absence of MLNs (Yamamoto et al. 2000; Alpan et al. 2001). In addition, CD47−/− mice, which have significantly less CD11b+ CD172a+ dendritic cells within MLNs, showed a reduction in the enterotoxin-induced IgA immunity in the gut (Westlund et al. 2012). The heterogeneity of dendritic cells within MLNs may play different roles in the induction of oral tolerance and in driving the systemic immune responses after vaccination, or intestinal stimulation with Toll-like receptor ligands (Onodera et al. 2009; Milling et al. 2010). These results suggested that there was a relationship between the suppression of ethanol-mediated IgA responses and the decrease in palmitoyl–ova transport to MLNs. Future studies should investigate the effects of ethanol on the cellular signaling and activation after orally administered antigens.

In contrast to the effects of ethanol on mucosal immunity against the palmitoyl-ova, systemic immunity was not affected by the treatment. A previous study suggested that M cells, Peyer's patches, and MLNs might only be involved in local gut responses to particulate antigens and that other cells might be involved in systemic responses to orally administered antigens (Alpan et al. 2001). This was supported by the roles of the spleen (Barsante et al. 2001; Oliveira et al. 2002) and liver (Li et al. 2004) in the development of systemic immunization, or tolerance to orally administered antigens. Thus, systemic immune responses remained unchanged because ethanol did not affect the levels of these antigens in these organs.

## CONCLUSION

Results showed that ethanol caused morphological and physiological changes that modify the absorption and mucosal lymphoid tissue distribution of orally administered particulate antigens. Changes in antigen biodistribution might play a role in the suppression of intestine-specific antibody production.

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