



## Oral-tolerance induction in diet-induced obese mice

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### Abstract

**Objective:** It is known that immune functions are altered in various ways by obesity. However, changes in the intestinal immune system resulting from obesity remain poorly understood. Oral tolerance is a system that suppresses antigen specific immune responses to orally administrated antigens. The intestinal immune system is intimately associated with the oral tolerance system, that acts to prevent allergic and inflammatory diseases. In this study we investigated the effect of obesity on induction of oral tolerance to ovalbumin (OVA) in an animal model of obesity. **Research methods and procedures:** Obese mice induced by a high fat diet and control mice were allowed free access for 3 days to a 1%-ovalbumin (OVA) solution in drinking water. After continuous feeding of the antigen, all the mice were immunized by two intraperitoneal injections of OVA administered 7 days apart. **Results:** In the control mice, induction of oral tolerance caused an increase in antigen specific IgG1 levels and a decrease in IgG2a levels. In contrast, the IgG1/IgG2a ratio was reversed in obese mice. OVA-specific IL-2 production was suppressed by antigen feeding in both the control and obese mice; however, suppression of OVA-specific IL-10 was observed only in the control mice. Although OVA-specific IgA and IgM were not affected by antigen feeding, the obese groups of mice had significantly lower titers of antibodies. **Discussion:** These findings suggest that obesity may affect induction of oral tolerance following antigen feeding and that these changes may be related to the inflammatory reaction.

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**Keywords:** Oral tolerance; Intestinal immune system; Diet-induced obese mice

### Introduction

The intestinal immune system has a unique role within the host defense, different from that of the general immune system (Crowe and Perdue, 1992), in that gut-associated lymphoid tissue has a role in the system. One of the functions of the intestinal immune system is the development of oral tolerance, a system that suppresses or induces responsiveness of antigen specific immune reactions to orally administrated antigens. Given the importance of this role, the system is seen as a new therapeutic strategy for treating allergic and inflammatory diseases (Weiner, 1997). Intestinal exposure to an antigen induces oral tolerance by a mechanism that may involve induction of regulatory T-cells or clonal deletion or anergy of antigen specific immune cells. The dose of antigen affects the immunological mechanism of oral tolerance, with low doses

generally inducing activation of regulatory T-cells, whereas high doses cause clonal deletion by apoptosis or anergy (Gregerson et al., 1993; Friedman and Weiner, 1994). These immunological mechanisms do not, however, make a clear distinction only on the basis of the antigen dose, implying that other complicated actions are involved in the development of oral tolerance (Burstein and Abbas, 1993).

Immune function is changed in various ways by obesity. It has been shown previously that obese humans and genetically obese animals have decreased numbers of immune cells in addition to impaired T-cell function and NK activity (Fink et al., 1996; Nieman et al., 1999; Tanaka et al., 1993; Fernandes et al., 1978; Mandel and Mahmoud, 1978; Candra, 1981; Tanaka et al., 1998). Changes in immune function as a result of obesity may be a major cause of the increased susceptibility to infection and several types of cancer seen in overweight individuals. Furthermore, numerous recent epidemiological studies have shown that the prevalence of atopy and asthma is higher in obese subjects than in subjects with normal body weight (Luder

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et al., 1998; von Mutius et al., 2001; Guerra et al., 2002; Mishra, 2004). While we have demonstrated previously that cytokine balance and antigen specific immune responses are altered in diet-induced obese mice (Mito et al., 2000; Mito et al., 2002), the changes in the intestinal immune system associated with obesity remain to be established. We speculate that one of the functions of the intestinal immune system, oral tolerance, may be affected by many factors including aging and nutritional conditions such as zinc deficiency or low protein diets (de Faria et al., 1998; Finamore et al., 2003; Satter et al., 2002). This study therefore investigated whether obesity affected induction of oral tolerance in diet-induced obese mice.

## Materials and methods

### Mice and diet

Four-week-old C57BL/6 mice were obtained from Oriental Yeast (Tokyo, Japan). The mice were maintained at 22 °C with a 12-h light:dark cycle. One group of mice was fed a high fat diet to induce obesity, and another group of mice was fed a standard diet as controls. The high fat diet consisted of 10.8% protein, 38.9% carbohydrate and 50.3% fat, whereas the standard diet consisted of 16.1% protein, 73.6% carbohydrate and 10.3% fat as % of total calories. These diets were manufactured by Oriental Yeast. The two groups of mice were fed their respective diets for 20 weeks, during which time they had free access to water and food.

### Induction of oral tolerance

After 20 weeks feeding, OVA was introduced into the drinking water, and provided ad libitum for 3 days to one-half of the mice from each diet group in order to induce oral tolerance. Both the obese and control mice consumed about 5 mL of OVA-containing water (1 mg/mL) per day, equivalent to an antigen intake of approximately 5 mg/day. This intake was not significantly different between the two groups. The other half of the mice in the obese and control groups were provided with water only. On the third consecutive day of OVA intake, all the mice were immunized by an intraperitoneal (i.p.) injection of 100 µg OVA absorbed to 3 mg alum in 0.5 mL phosphate-buffered saline (PBS). Seven days later, the mice received a further identical i.p. immunization. Five days after this second immunization, all the mice were sacrificed by cervical dislocation, and samples of blood and spleen collected.

### Measurement of serum concentration of antigen specific antibodies

Serum was obtained by centrifugation. A standard enzyme-linked immunosorbent assay (ELISA) was used to determine OVA-specific antibody titers. Briefly, micro plates were coated with OVA (20 µg/mL in NaHCO<sub>3</sub> buffer, pH 9.6) and plated overnight at 4 °C. After incubation of the sera in the coated plates, biotinylated anti-mouse IgG1, IgG2a, and IgM (Zymed Laboratories, San Francisco, CA), or IgA (CALTAC, Burlin-

game, CA, UK) antibodies were added. The reactions with all the antibodies were then detected by peroxidase-conjugated streptavidin (DAKO, Kyoto, Japan), using TMB as the substrate.

### Preparation of splenocytes

Single cell suspensions of splenocytes in RPMI-1640 medium (NISSUI, Tokyo, Japan) were made by passing a spleen homogenate through a stainless steel mesh, followed by filtration through a 250-µm nylon mesh. The cell suspensions were then washed and diluted in RPMI-1640 medium containing 5% heat-inactivated fetal calf serum (JRH, Lenaxa, Australia), L-glutamine (2 nmol/L; GIBCO, Grand Island, NY, USA), penicillin (100 U/mL; GIBCO), and streptomycin (100 µg/mL; GIBCO). The number of cells was adjusted to a density of  $4 \times 10^6$  cells/mL and cultured in the presence of OVA (100 µg/mL) stimulation for 48 h at 37 °C in a 5% CO<sub>2</sub> atmosphere.

### Measurement of antigen specific cytokine by splenocytes

ELISAs were performed on the culture supernatants of OVA-stimulated splenocytes to measure production of the cytokines IL-2, IL-4 and IL-10. The supernatants were collected after incubation for 48 h, centrifuged and stored at -30 °C until analysis. An ELISA system, Duo set (R and D system, Inc., Minneapolis, MN, USA), was used for these assays according to the manufacturer's instructions.

### Statistical analysis

Results are expressed as means ± SE. The data were analyzed by Student's *t* test with differences with a *P* value < 0.05 being considered significant.

## Results

### Body weight

Eight mice in each of the obese and control groups received 1%-OVA dissolved in their drinking water (Oral) or water (Immune) for 3 consecutive days.

The obese-oral (tolerance+) and obese-immune (tolerance-) groups had a significant increase in final body weight compared with both the control groups (Table 1). Induction of oral tolerance did not affect body weight.

Table 1  
Body weight measurements in all the groups of mice

| Group               | Obese                   |                         | Control    |            |
|---------------------|-------------------------|-------------------------|------------|------------|
|                     | Oral                    | Immune                  | Oral       | Immune     |
| Initial body weight | 16.3 ± 0.4              | 16.6 ± 0.3              | 17.0 ± 0.3 | 16.6 ± 0.3 |
| Final body weight   | 35.8 <sup>a</sup> ± 1.9 | 37.6 <sup>a</sup> ± 1.5 | 23.0 ± 0.3 | 24.0 ± 0.5 |

Mean ± SE. Each group contained eight mice.

<sup>a</sup> Vs. control-oral or immune group, *P* < 0.001.

### OVA-specific IgG1 and IgG2a in sera

In the control group, the levels of OVA-specific IgG1 were increased significantly in the control-oral group compared with the control-immune group ( $P<0.01$ ) (Fig. 1). In contrast, no significant difference was observed between the obese-oral and obese-immune groups. The levels of OVA-specific IgG1 were also lower in the obese-oral group than in the control-oral group ( $P<0.01$ ). OVA-specific IgG2a levels tended to be lower in the control-oral group compared with the control-immune group (Fig. 1), whereas in the obese mice, the levels were similar in the oral and immune groups. Fig. 1 also shows that the OVA-specific IgG1/G2a ratio was significantly greater in the control-oral group compared with the control-immune group ( $P<0.05$ ) (Fig. 1). In contrast, in the obese mice, the oral-group had a tendency to have lower OVA-specific IgG1/G2a ratios than the immune-group.

### OVA-specific IgA and IgM in sera

Although induction of tolerance did not affect the serum levels of OVA-specific IgA and IgM in either the control or obese mice, the obese groups had significantly lower levels of antibody titers (IgA: obese-oral vs. control-oral,  $P<0.05$ , obese-immune vs. control-immune,  $P<0.05$ ; IgM: obese-oral vs. control-oral,  $P<0.01$ , obese-immune vs. control-immune,  $P=0.069$ ) (Fig. 2).

### OVA-specific cytokine (IL-2, IL-10, IL-4) production by splenocytes

Oral-tolerance induction induced suppression of OVA-specific IL-2 in both the obese and control groups ( $P<0.05$ ).

The obese-immune group had lower levels of IL-2 compared with the control-immune group ( $P<0.01$ ) (Fig. 3). No significant difference was observed in OVA-specific IL-4 between all the groups of mice (Fig. 3). Production of OVA-specific IL-10 was decreased significantly in the control-oral group compared with the control-immune group ( $P<0.05$ ), although no such difference was observed between the obese-oral and obese-immune groups (Fig. 3).

### Discussion

It has been reported that cellular immunity is impaired in both genetically obese animals and obese human subjects (Fink et al., 1996; Nieman et al., 1999; Tanaka et al., 1993; Fernandes et al., 1978; Mandel and Mahmoud, 1978; Candra, 1981; Tanaka et al., 1998). However, to date, changes in the intestinal immune system caused by obesity have not been studied extensively. We have reported previously a variety of changes in cytokine production by splenocytes from diet-induced obese mice (Mito et al., 2000; Mito et al., 2002). As these mice are a good model of human obesity, we used this animal model in the present study to investigate changes in the oral tolerance system.

Gastric gavage and continuous exposure of antigen in water taken orally are commonly used methods for administering antigens with the aim of inducing tolerance. In this study, oral tolerance was induced by continuous feeding of a 1%-OVA solution in drinking water similar to that described in previous studies (Russo et al., 2001; Faria et al., 2003). It has been reported that this method leads to selective tolerization of the Th1 response and up-regulation or predominance of the Th 2

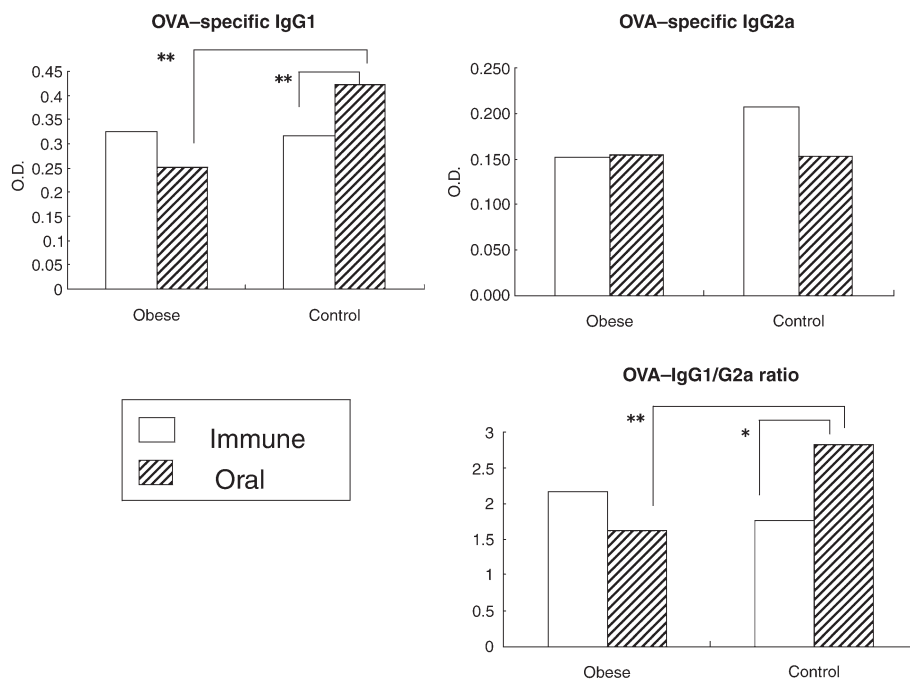


Fig. 1. Serum levels of OVA-specific IgG1 and IgG2a. All mice received i.p. immunization with OVA after drinking a 1%-OVA solution (Oral) or Water (Immune) for 3 days. \* $P<0.05$ , \*\* $P<0.01$ .

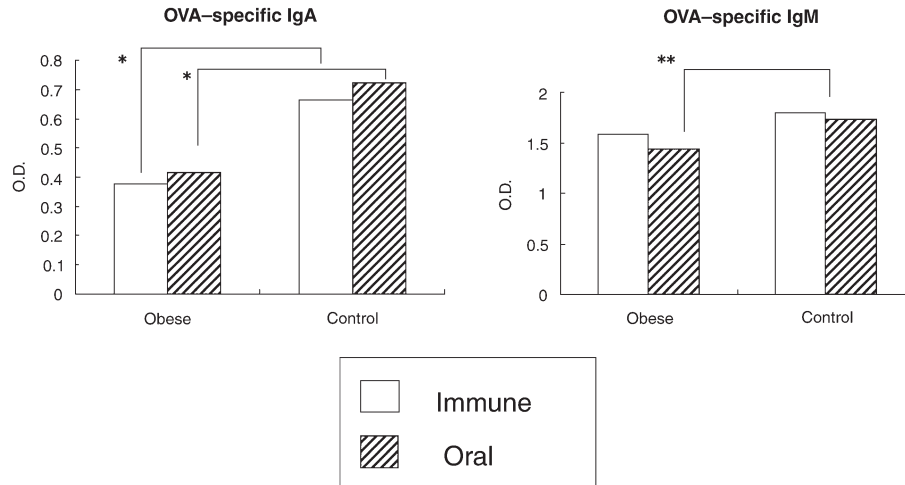


Fig. 2. Serum levels of OVA-specific IgA and IgM sera. All mice received i.p. immunization with OVA after drinking a 1%-OVA solution (Oral) or Water (Immune) for 3 days. \* $P < 0.05$ , \*\* $P < 0.01$ .

response. In the present study, the OVA-fed control mice showed suppression of IgG2a (Th1) and increased IgG1 (Th2) compared with OVA-unfed control mice, results that are consistent with earlier reports on oral tolerance (Melamed and Friedman, 1994). However, we found that the obese mice had different patterns of immune response to the antigen feeding compared with control animals. For example, OVA-fed obese mice had decreased IgG1 levels compared with OVA-unfed obese mice, whereas IgG2a levels were similar in the two groups of mice. On the other hand, serum levels of OVA-specific IgM and IgA, while not affected by antigen feeding in either the obese or control mice, were significantly lower in

obese animals compared with controls. These observations suggest that induction of oral tolerance in the intestinal immune system may be affected by obesity.

In this study we also investigated the production of antigen specific cytokines by cultured splenocytes. We found OVA-specific IL-2 production was suppressed, while OVA-specific IL-4 production remained unchanged following OVA-feeding in both obese and control mice. These results support the concept that induction of oral tolerance is an effect of Th2 response predominantly (Melamed and Friedman, 1994; Gilbert et al., 1990). However, we showed that OVA-specific IL-10 production was decreased in OVA-fed control mice compared

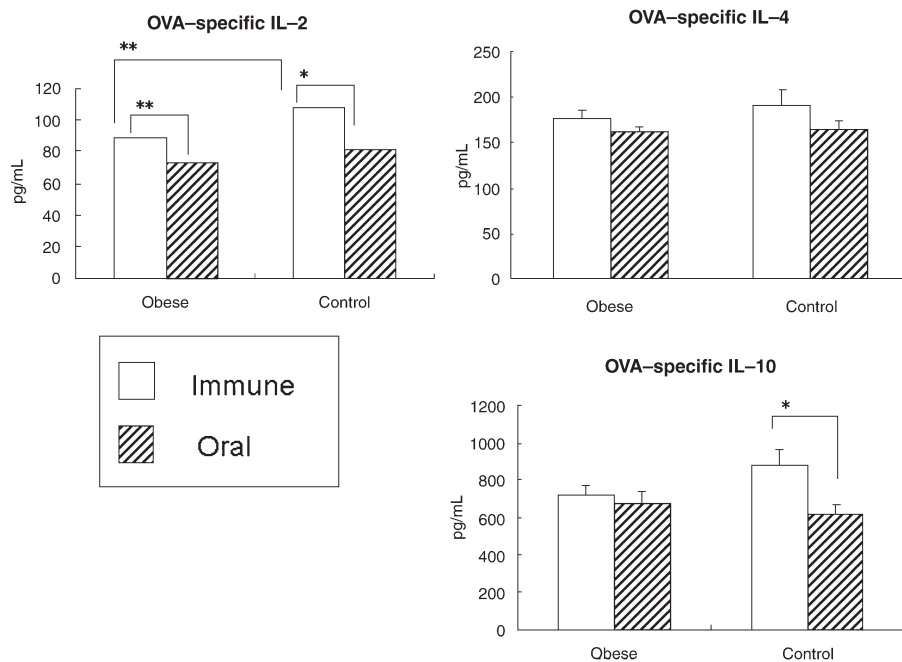


Fig. 3. Production of OVA-specific cytokines, IL-2, IL-4, and IL-10 in the supernatant of splenocytes cultured for 48 h. All mice received i.p. immunization with OVA after drinking a 1%-OVA solution (Oral) or Water (Immune) for 3 days. \* $P < 0.05$ , \*\* $P < 0.01$ .

with OVA-unfed control mice, although no such difference was observed in the obese groups. These changes in cytokine patterns do not, however, explain the altered antibody titers that occur with induction of oral tolerance in obesity. As anergy by oral tolerance is induced readily in Th1 type clones, but not in Th2 clones, further studies are required to analyze the effect of obesity on T-cell subsets and investigate the difference of strains using Th2-dominant Balb/c mice in addition to C57BL/6 mice.

The chemokine, monocyte chemo-attractant protein-1 (MCP-1), has been shown to regulate oral tolerance induced by Th2-like responses (Karpus and Ransohoff, 1998; Gonnella et al., 2003). However, both human and animal models of obesity have been shown to have high serum levels of MCP-1, as not only immune cells but also hypertrophic adipocytes may produce MCP-1 (Weisberg et al., 2003; Wellen and Hotamisligil, 2003). Accordingly, the elevation of systemic levels of MCP-1 we confirmed in our diet-induced obese mice may be related to changes in intestinal immune function. Further studies measuring MCP-1 expression in the intestinal mucosa in obese animals are required to investigate this possibility. IFN- $\gamma$  is another factor that is related to oral tolerance, with studies showing that treatment of mice with IFN- $\gamma$  prior to antigen feeding results in blocking of oral-tolerance induction (Zhang and Michael, 1990). One possible mechanism to explain this suppressive effect of IFN- $\gamma$  is induction of iNO synthase as there is evidence that an absence of functional iNO synthase augments the efficacy of tolerance induced by high dose antigen feeding (Kahn et al., 2000). We reported recently that splenocytes from diet-induced obese mice had increased IFN- $\gamma$  production. Taken together these results indicate that changes in immune function in diet-induced obesity may affect induction of oral tolerance. In this regard we suggest that further studies are required to investigate GALT function in obesity. It also would be necessary to examine differences in lymphocyte populations in the intestine.

Obese humans and diet-induced obese mice both have increased serum leptin levels and exhibit leptin resistance in the hypothalamus (Mito et al., 2002; Considine et al., 1996). Leptin is an adipocyte derived hormone that has numerous roles in immune regulation, such as prevention of thymocyte atrophy during starvation, inducing increased T-cell proliferation and Th1-type cytokine production, and activation of monocyte and natural killer cells (Loffreda et al., 1998; Lord et al., 1998; Martin-Romero et al., 2000; Tian et al., 2002). It is known that immune cells also have leptin receptors and it has also been reported that leptin protects against apoptosis of immune cells by inducing bcl-xL (Fujita et al., 2002). Apoptosis of antigen specific T-cells in the thymus, spleen and peripheral lymph nodes is induced by oral administration of antigen, and is one of the important steps in the induction of oral tolerance (Ueda et al., 2001). Although the role of leptin in peripheral tissues, including immune cells, is not fully understood, we have reported previously that leptin sensitivity of immune cells may be altered by obesity (Mito et al., 2004). It is therefore possible that leptin may be a factor that affects immune function in obesity and it is interesting to note that leptin receptors are also expressed in intestinal tissues (Morton

et al., 1998; Guilmeau et al., 2004). Possible mechanisms of oral tolerance, such as clonal deletion of T-cells by apoptosis or activation of regulatory T-cells, may be affected by abnormal leptin concentrations or function in obesity. It also would be necessary to examine differences in lymphocyte populations in the intestine.

Oral administrations of antigen activate the intestinal immune system, an example of which is the production of antigen specific secretory IgA (McGhee et al., 1992). sIgA is an important factor in the oral tolerance system, as it has anti-inflammatory and antigen exclusion actions in the intestine (Walker and Hong, 1973; Walker et al., 1975). In our study, we detected sIgA in the fecal samples obtained from the OVA-fed control mice, but little or no antibody was found in samples from either OVA-unfed control mice or both the obese groups of animals (data not shown). IgA in the sera is produced from bone marrow and other hematopoietic cells, whereas sIgA is produced by cells in mucosal-associated lymphoid tissue. Although it is difficult to detect sIgA as it is present at very low concentrations, it has been reported that sIgA activity may reflect serum IgA activity (Kobayashi et al., 1987). In our study serum IgA levels were significantly lower in diet-induced obese mice. Accordingly sIgA producing mechanism in response to the oral antigen may be impaired in obesity, resulting in the changes in oral-tolerance induction we observed in the obese mice. The results of IgA and IgM in the present study, however, seemed to be relevant to obesity, rather than oral-tolerance induction.

In conclusion, this study indicates that obesity may affect induction of oral tolerance following feeding of high doses of antigen. It is possible these changes may be related to the increased prevalence of allergic and inflammatory condition associated with obesity. Although oral-tolerance induction by antigen feeding as a new immune therapy would be expected to improve both allergy conditions and autoimmune diseases, it is possible this therapy may not be effective in obese subjects.

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