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Research paper

Antigen administration by continuous feeding enhances oral tolerance and leads to long-lasting effects

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ABSTRACT

The ability to avoid inflammatory responses to dietary components and microbiota antigens in the gut mucosa is achieved by a mechanism termed oral tolerance. This phenomenon is crucial to maintain the physiological immune activity in the gut and to prevent inflammatory disorders such as food allergy and inflammatory bowel diseases. Moreover, orally administered antigens induce regulatory cells that control systemic inflammatory responses as well. Given its specific, systemic and long-lasting effects, oral tolerance represents a promising approach for immunotherapies that aim to modulate inflammatory and autoimmune diseases. However, there are different protocols of feeding for induction of oral tolerance, and they have an impact in tolerance efficiency and length. Herein, we present and discuss different experimental feeding protocols and how they influence the outcome of oral administration of antigens.

1. Introduction

The intestine is the largest surface area of the human body (Mestecky et al., 2005); it also bears an abundant lymphoid tissue that is continuously contacting a plethora of environmental stimuli such as dietary and microbiota antigens (Veldhoen and Brucklacher-Waldert, 2012; Randall and Mebius, 2014). The default outcome from this daily stimulation is the maintenance of physiological non-inflammatory state of homeostasis. The mechanisms involved in such state are also able to generate specific tolerance to ingested antigens, a phenomenon named oral tolerance. Although originated in the gastrointestinal tract, oral tolerance has systemic effects inhibiting the production of specific serum antibodies, cell proliferation and cytokine production in other tissues (Faria and Weiner, 2005).

Since the pioneer studies reporting that eating a certain protein inhibits immune responses to the same antigen after immunization (Richman et al., 1978), a great advance in the understanding of oral tolerance has been achieved. Several mechanisms have been shown to be involved in the phenomenon depending on a unique set of mucosal organized structures and lymphoid compartments cooperating to generate tolerogenic responses. Special features of gut macrophages and dendritic cells are essential to collect and present gut-derived antigens in a non-immunogenic fashion (Mazzini et al., 2014). The production of anti-inflammatory cytokines such as IL-10 and TGF-beta and the induction of $CD4^+$ regulatory T cells expressing the transcription factor Foxp3 are also critical for oral tolerance induction (Mucida et al., 2005).

Despite being a promising approach for immunoregulatory interventions and having successfully prevented a number of inflammatory and autoimmune diseases in animal models, oral tolerance has not been effectively translated to a clinical setting yet (Weiner et al., 2011).

Therefore, is imperative to consider the full requirements for boosting the tolerogenic mechanisms induced by mucosal antigen exposure and improve protocols and regimens for oral

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tolerance induction and maintenance. A number of studies have shown that the frequency as well as form of the antigen administration by the oral route is a critical factor for oral tolerance induction. Experiments in mice, rats and guinea pigs (Heppell and Kilshaw, 1982; Saklayen et al., 1984; Peng et al., 1989) showed that multiple feeding by gavage were more effective than a single feeding of antigen to induce oral tolerance to inflammatory immune responses and autoimmune disease models (Higgins and Weiner, 1988; Thompson and Staines, 1990; Melamed et al., 1996). Our group and others have also demonstrated that continuous feeding of the antigen is more efficient than single or multiple feedings by gavage in inducing suppression of inflammatory disease models (Faria et al., 1998; Russo et al., 1998; Marth et al., 2000). Continuous feeding correlates with enhanced production of TGF-beta and IL-10 (Marth et al., 2000; Faria et al., 2003). These studies have presented convincing data on the suppressive effects of single, multiple and continuous regimens of antigen feeding. However, there is no systematic study available comparing side by side the two most used regimens of feeding, gavage (intragastric administration of antigen) and continuous feeding (antigen administration in the drinking water), in inducing durable oral tolerance under distinct immunological settings and in different mouse strains. Herein, we present and discuss our results regarding such comparative study. We also present methodological details on the two protocols of feeding that are relevant for their effects.

2. Materials and methods

2.1. Animals

Eight- to twelve-week-old male or female BALB/c, C57BL/6 and B6D2F1 mice were supplied by the Animal Facility of Universidade Federal de Minas Gerais (UFMG). All animal procedures were approved by the local Ethical Committee for Animal Experimentation (CETEA-UFMG). Mice were kept in the conventional experimental animal facility of Laboratório de Imunobiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Experimental groups contained 4–16 mice.

2.2. Oral tolerance induction

The antigen used was ovalbumin (OVA, grade III, Sigma, St. Louis, MO). Single and multiple feedings were performed by daily intragastric administration (gavage) of 20 mg OVA in 0.2 ml saline using a round-tip 18-gauge stainless animal feeding needle (Thomas Scientific, Swedesboro, NJ). Continuous feeding refers to the administration of the antigen (at a concentration of 4 mg/ml) in the drinking water for 24 h. Control groups received either gavages of saline or bottles containing water.

2.3. Antigen and immunizations

Seven days after oral treatment mice were injected intraperitoneally (i.p.) with 10 μg OVA plus 3 mg of $Al(OH)_3$ as adjuvant in 0.2 ml of saline. A booster with 10 μg Ova in saline was given i.p. 14 days later and 7 days thereafter, mice were bled under anaesthesia from the axilliary plexus and serum was collected for antibody assays. For delayed-type hypersensitivity (DTH) assay, mice received 100 μg OVA emulsified in complete Freund´s adjuvant (CFA) containing 50 μg *Mycobacterium tuberculosis* H37 RA (DIFCO, Detroit, MI) subcutaneously (s.c.) in the base of the tail 7 days after oral treatment. Mice were challenged with 30 μl 1% thermally aggregated OVA (600 μg OVA/mouse after 2 min treatment at 100 °C) in the right footpad 14 days later. Animals were injected with 30 μl saline in left footpad for control purposes. Increase in footpad thickness was measured 24 h later using a caliper.

2.4. Antibody assays

2.4.1. Anti-OVA total Ig and Anti-OVA IgG1

Anti-OVA antibody titers were determined by enzyme linked immunosorbent assay (ELISA). Briefly, 96-well plates (Nunc, Roskild, Denmark) were coated overnight with 20 μg/ml OVA solution in sodium carbonate buffer, pH 9.6, at 4 °C. Plates were washed with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature (RT) with PBS containing 0.25% casein. Plates were incubated for 1 h at 37 °C with eight dilutions of mouse serum samples starting at 1/100 in PBS-casein. They were washed and incubated with either horseradish-peroxidase(HRP)-labeled goat anti-mouse IgG1 or HRP-labeled goat anti-mouse Ig (Southern Biotechnology, Birmingham, AL) for 1 h at 37 °C. Plates were washed, and incubated in the dark with H_2O_2 in the presence of orthophenylenediamine (OPD, Sigma) in sodium citrate buffer; pH 5.0 for 20 min. Reaction was stopped with 20 μl of 2N H_2SO_4. Optical density was measured using an automatic ELISA reader at 492 nm. Results were calculated by running sum of ODs of serum dilutions between 1:100 and 1:12,800 of individual mice. This method represents more precisely antibody titers as previously described by our group (Carvalho et al, 1994; Verdolin et al., 2001). Alternatively, the anti-Ova IgG1 concentration was obtained by interpolating a standard curve obtained by different concentrations of a mouse monoclonal anti-Ova IgG1 antibody (monoclonal OVA-14, Sigma).

2.4.2. Anti-OVA IgE

Anti-OVA antibody titers were determined by a modified enzyme linked immunosorbent assay (ELISA) based on the method previously described by Facincone et al. (1997). Briefly, 96-well plates (Nunc, Roskild, Denmark) were coated overnight with rat anti-mouse IgE-UNLB (Southern Biotechnology, Birmingham, AL) in sodium carbonate buffer solution, pH 9.6, at 4 °C. Plates were washed with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with PBS containing 0.25% casein. Plates were incubated for 1 h at room temperature with undiluted mouse serum samples, further washed and incubated with a PBS solution containing 20 μg/ml biotinylated ovalbumin and 0.25% casein for 1 h at room temperature. Plates were washed and incubated with streptavidin-HRP (Southern Biotechnology, Birmingham, AL) solution at room temperature for 1 h. Then plates were washed and incubated in the dark with H_2O_2 in the presence of orthophenylenediamine (OPD, Sigma) in sodium citrate buffer, pH 5.0, for 20 min. Reaction was stopped with 20 μl of 2N

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