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Aging correlates with reduction in regulatory-type cytokines and T cells in the gut mucosa

Andrezza F. Santiago[a], Andréa C. Alves[a], Rafael P. Oliveira[a], Raphaela M. Fernandes[a], Josiely Paula-Silva[a], Frankcineia A. Assis[a], Cláudia R. Carvalho[b], Howard L. Weiner[c], Ana Maria C. Faria[a,*]

[a] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil
[b] Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil
[c] Center for Neurologic Diseases, Brigham & Women's Hospital, Harvard Medical School, Boston, MA, USA

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ABSTRACT

Aging is reported to be associated with decline in oral tolerance induction, which is initiated at the intestinal mucosal surface. Herein, we examined the effect of aging in T cells and cytokines at the intestinal mucosa that might be involved in oral tolerance induction. Frequencies of regulatory-type IEL subsets such as TCRγδ$^+$ and TCRαβ$^+$CD8αα$^+$ were lower in aged mice. Mucosal CD4$^+$CD25$^+$Foxp3$^+$ and CD4$^+$LAP$^+$ T cells increased with aging but activated CD44$^+$CD4$^+$ mucosal T cells also augmented. Production of TGF-β and IL-10 in the small intestine of old mice was reduced. Moreover, the ability of mucosal dendritic cells of aged mice to stimulate TGF-β secretion and differentiation of CD4$^+$LAP$^+$ T cells in co-culture studies also declined with aging. Reduction in these regulatory-type cytokines and T cells may help to explain the decline in susceptibility to oral induction during aging. However, not all mucosal regulatory elements were altered by aging and CD4$^+$CD25$^+$Foxp3$^+$ T cells were especially resistant to changes. Persistence of some mechanisms of regulation may play a critical role in maintaining mucosal homeostasis during aging.

Introduction

The gut mucosa is a major route for foreign antigens to contact the immune system. A large and regular amount of dietary antigens reach the gut daily, and a continuous exposure to the autochthonous microbiota provides additional stimulation to the abundant lymphoid tissue located in the intestinal mucosa (Brandtzaeg 1998). This daily antigenic contacts play an important role in the development of the immune system after weaning (Menezes et al. 2003). Antigenic contact initiated by oral route is known to induce oral tolerance, a state of systemic suppression of specific immune responses to subsequent parenteral injections of the same antigen (Vaz et al. 1977; Mowat 2003; Faria and Weiner 2005). Many subsets of lymphocytes with regulatory phenotype such as CD4$^+$LAP$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ have been described to play a critical role in oral tolerance induction (Faria and Weiner 2005; Curotto de Lafaille et al. 2008). Intraepithelial cells seem also to be involved in oral tolerance induction due to their ability to produce non-inflammatory and regulatory cytokines such as TGF-β and IL-10 which play an important role in the gut homeostasis (Ke et al. 1997; Saurer and Mueller 2009).

Oral tolerance is one of many immune functions altered by the aging process. In fact, aging brings about several changes in the immune system. It affects drastically the T cell compartment, cytokine production, antigen-specific antibody responses (Linton and Dorshkind 2004; Speziali et al. 2009; Santiago et al. 2008) and the composition of lymphoid organs such as gut Peyer's patches (Kato et al. 2003). Interestingly, development of aging alterations occurs earlier in the mucosal immune system than in the systemic immune compartment (Koga et al. 2000). Our group has previously shown that the two main immunological events initiated at the gut surfaces, IgA production and oral tolerance induction, are differentially affected by the aging process. Susceptibility to oral tolerance decreases with age. Mice that is susceptible to oral tolerance induction by a single feeding of antigen at 8 weeks of age become less susceptible at 24 weeks and totally refractory at 70 weeks of age (Faria et al. 1993). Only a regimen of continuous feeding is able to render 70-week-old mice tolerant to orally administered antigen (Faria et al. 1998, 2003). On the other hand, production of

Abbreviations: DCs, dendritic cells; IEL, intraephitelial cells; LP, lamina propria; MLN, mesenteric lymph nodes; OVA, ovalbumin; PP, Peyer's patches; S-IgA, specific secretory IgA.

* Corresponding author. Tel.: +55 31 3499 2640; fax: +55 31 3499 2640.
E-mail address: afaria@icb.ufmg.br (A.M.C. Faria).

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secretory IgA (S-IgA), the most abundant immunoglobulin at mucosal surfaces, is unaltered in old mice (Santiago et al. 2008).

Few accounts are available evaluating immunological parameters such as cell types and cytokine production at the mucosal surfaces during the aging process. Since many regulatory-type elements exist in the gut environment, changes in these cellular and molecular components may be involved in the age-related decline of local immune functions such as oral tolerance induction. Among gut-associated T cells, there seem to be a decrease in $CD45^+$ T cells as well as an increase in intestinal $NK1.1^+$ T cells and double-positive $CD4^+CD8^+$ IEL population of aged mice (Ishimoto et al. 2004; Hayashi et al. 2009). Aging-related alterations on dendritic cell (DC) phenotype and function have also been investigated, but the results are still controversial. One study demonstrated that DCs derived from peripheral blood of humans do not show significant changes in phenotype or function with aging (Steger et al. 1996). Another report did not find changes in the percentage on spleen DC expressing CD80 and CD86 in studies conducted using senescence-accelerated prone mice (Haruna et al. 1995). Simioni et al. (2010) observed an age-related reduction in CD86 expression in spleen DCs, but not in the frequency of cells expressing this molecule.

In addition to the paucity of studies that addressed the effects of aging in the mucosal associated lymphoid tissue, the impact of aging in different compartments and regions of the gut-associated lymphoid compartment is not known. Our aim in this study was to perform a systematic evaluation of several regulatory-type cells and cytokines usually present in this tissue at different ages to study the impact of aging in function of mucosal associated lymphoid tissue. Our hypothesis is that putative alterations in immune elements in the gut may help to explain the decreased susceptibility to oral tolerance induction during aging.

Materials and methods

Animals

Female TCR OVA-specific transgenic (DO11.10) and BALB/c mice at ages ranging from 2 to 24 months were obtained from Centro de Bioterismo (CEBIO, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil) and maintained in our experimental animal facility throughout the experiments. Mice were kept in microisolators; autoclaved diet and water were offered *ad libidum*. All animal procedures were approved by the local ethical committee for animal research (Protocol # 115/2007, CETEA-UFMG, Brazil).

Analysis of Ig isotypes by ELISA

Levels of total immunoglobulins were determined by ELISA. Briefly, 96-well plates (NUNC, Roskilde, Denmark) were coated with 0.1 μg goat anti-mouse UNLB antibody, in coating buffer pH 9.8 overnight. Wells were washed and blocked with 200 μl of PBS contain 0.25% casein for 1 h at room temperature. Sera were added to the plate and incubated for 1 h at room temperature, plates were washed, then peroxidase–streptavidin goat anti-mouse or rat anti-goat (Southern Biotechnology, Birmingham, AL, USA) 1:15,000 was added, and plates were incubated for 1 h at 37 °C. Color reaction was developed at room temperature with 100 μl/well of orthophenylenediamine (1 mg/ml) (Sigma), 0.04% H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 μl/well of 2 N H_2SO_4. Absorbance was measured at 492 nm by an ELISA microplate reader (Bio-Rad Model 450, Hercules, CA, USA).

Histomorphometric analysis

Small intestine was removed and immersed in 10% formaldehyde buffer; tissues were further dehydrated with alcohol-containing solutions using an automatic tissue processor (Titertek, Huntsville, AL, USA). Gut tissues were then included in paraffin and 4 μm transverses sections obtained by Spencer microtome (Spencer Scientific Co, Derry, NH, USA). Tissues were stained with eosin and hematoxylin and morphologic profile determined using an Olympus microscope. (Olympus, Center Valley, PA, USA). Villous length was measured using millimeter laminule. IEL cells numbers were calculated by manually counting cells in 10 villous and expressed as ratios of IEL/100 epithelial cells as described by Ferguson and Murray (1971).

Cell preparation and cytokine assay

Small intestine was separated into duodenum, proximal jejunum, distal jejunum and ileum and placed in buffer solution (1 ml/g) containing 10,000 UIC/ml of aprotinin. Tissue fragments were homogenized and centrifuged for 15 min 600 × *g* at 4 °C. Supernatants were collected for cytokine assay. Plates were coated with purified monoclonal antibodies reactive with cytokines IL-4, IFN-γ, IL-10 and TGF-β (BD-Pharmingen, San Jose, CA, USA) overnight at 4 °C. In the following day, wells were washed and supernatants were added and plate was incubated overnight at 4 °C. In the third day, biotinylated monoclonal antibodies against cytokines are added and plate was incubated for 1 h at room temperature. Color reaction was developed at room temperature with 100 μl/well of orthophenylenediamine (1 mg/ml), 0.04% H_2O_2 substrate in sodium citrate buffer. Reaction was interrupted by the addition of 20 μl/well of 2 N H_2SO_4. Absorbance was measured at 492 nm by an ELISA reader (Bio-Rad Model 450). Cytokines were also determined in the supernatants of co-cultures of T and dendritic cell (DC)-enriched cells using the same methodology.

T cell and DC separation

Spleen T cell populations were obtained from 8-week-old naïve DO11.10 transgenic mice. Spleen and mesenteric lymph nodes DC population were obtained from young (2-month-old) and aged (12-month-old) BALB/c mice. T- and DC-enriched populations were isolated using MACS micro beads (anti-Thy1 and anti-CD11c) and LS MACS Column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer instructions. Enriched cell suspensions were monitored by flow cytometry. DC preparations contained approximately 60% $CD11c^+$ cells and T cell contained approximately 95% $CD3^+$ cells. T $CD4^+$ cells from naïve DO11.10 mice were 80–90% KJ1.26 positive.

Lymphocyte preparation and flow cytometry analysis

Small intestines and mesenteric lymph nodes were removed from euthanized mice, flushed with cold calcium and magnesium free HBSS. Payer's patches and fat were then removed from the tissues that were incubated in HEPES-containing HBSS medium (2% HEPES buffer, 1% penicillin/streptomycin and 0.05% gentamicin). Mesenteric lymph nodes were smashed and passed through a 70 μm cell strainer (BD Falcon). For intraepithelial lymphocytes (IEL) cells isolation, small intestine was washed, diced and incubated in IEL medium (RPMI containing 2% FBS, 2% HEPES buffer, 1% penicillin/streptomycin and 0.5% gentamicin) for 40 min at 37 °C in a shaker (150 rpm). Cells were filtered through a 70 μm cell strainer and supernatant containing IEL fraction kept in ice. For lamina propria (LP) cells isolation, cells were incubated with 100 μg/ml collagenase II for 40 min at 37 °C in a shaker (150 rpm), smashed