Journal of Neuroimmunology 276 (2014) 47–57

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Impaired responses to gliadin and gut microbes of immune cells from mice with altered stress-related behavior and premature immune senescence

G. De Palma [a], C. Vida [b], A. Santacruz [a], N.M. De Castro [b], M. De la Fuente [b], Y. Sanz [a,*]

[a] Microbial Ecology, Nutrition & Health Research Group, Institute of Agrochemistry and Food Technology, National Research Council (IATA-CSIC), Valencia, Spain
[b] Department of Physiology, Faculty of Biology, Complutense University of Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 16 April 2014
Received in revised form 3 August 2014
Accepted 6 August 2014

Keywords:
Stress-related behavior
Celiac disease
Intestinal bacteria
Leukocytes
Aging

ABSTRACT

Stress is associated with impaired communication between the nervous and immune systems leading to immunosenescence and increased disease risk. We investigated whether leukocytes from mice with altered stress-related behavior and premature immunosenescence, as well as from chronologically aged mice differently responded *ex vivo* to celiac disease (CD) triggers (gliadin) and intestinal bacteria by ELISA and flow cytometry and differed in microbiota composition. We found that altered stress-related behavior and premature immunosenescence led to alterations in T lymphocytes and cytokine release of immune cells basally and in response to peptic fragments of gliadin and commensal and pathogenic bacteria, possibly increasing susceptibility to CD in adulthood.

1. Introduction

Communication between the nervous and immune systems ("the neuro-immune axis") is essential to health (Besedovsky and Del Rey, 2007), thus its disruption plays a fundamental role in age-related physiological decline and contributes to increasing morbidity and mortality (De la Fuente, 2008; Gemma, 2010).

Stress and inadequate responses to environmental stressors are related to dysfunctional nervous–immune system communication, which also contributes to accelerating aging and onset of age-related diseases (Bauer, 2008; Costa-Pinto and Palermo-Neto, 2010; Yirmiya and Goshen, 2011). Hyper-reactivity to stressors in mice and rats is genetically linked to a shorter lifespan, higher oxidative and inflammatory stress, acceleration of neurodegenerative changes in the brain and premature immune senescence (Viveros et al., 2007; Vida and De la Fuente, 2013). Moreover, the immunological changes observed in chronological aging may be closely related to those observed in psychological stress. In fact, the changes in cellular trafficking as well as in cell-mediated immunity observed in aging are similarly found following stress or chronic glucocorticoid exposure (Bauer, 2005, 2008).

Aging is also associated with alterations in neuroendocrine responses to stress, such as an altered function of the hypothalamus–pituitary–adrenal axis (Orentreich et al., 1984), which is crucial for the regulation of stress and anxiety-related responses. Thus, there is also an age-related impairment of the stress response (Bauer and De la Fuente, 2008). In addition, rodent longevity appears to be inversely related to the intensity of their behavioral and neuroendocrine responses to stressful stimuli (Dellu et al., 1994), and reduced longevity could be caused by accelerated age-dependent neurodegeneration (Gilad and Gilad, 1995). In this context, several studies carried out by De la Fuente et al. reported that inter-individual differences among members of outbred Swiss and inbred BALC/c mouse populations, both male and female, were related to their ability to explore a simple T-maze. Animals exhibiting immobility or "freezing behavior" in this new environment failed the exploratory test and showed a worse immune function than those mice performing the test correctly (De la Fuente et al., 1998; Viveros et al., 2001, 2007; Guayerbas et al., 2002a,b,c; Vida and De la Fuente, 2013). The animals with premature immunosenescence also showed a premature impairment of the nervous system (at neurochemical and behavioral levels), with a decreased adaptive response to stressful situations, as well as increased emotional reactivity and anxiety, high oxidative and inflammatory stress in their immune cells (and in other cells) and a shorter lifespan. Thus, a

Abbreviations: ANP, adult NPAM; AP, adult PAM; APCs, antigen presenting cells; CD, celiac disease; CFU, colony-forming unit; MRS, De Man, Rogosa and Sharpe; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MLNs, mesenteric lymph nodes; NPAM, non-prematurely aged mice; OP, old PAM; ONP, Old NPAM; PBS, phosphate-buffered saline; PAM, prematurely aged mice; qPCR, quantitative real-time PCR; TNF, tumor necrosis factor; VRBD, violet red bile dextrose.

* Corresponding author at: IATA-CSIC, Avda. Agustín Escardino, 7. 46980 Paterna, Valencia, Spain. Tel.: +34 963900022; fax: +34 963636301.
E-mail address: yolsanz@iata.csic.es (Y. Sanz).

http://dx.doi.org/10.1016/j.jneuroim.2014.08.007
0165-5728/

murine model was established of premature aging, based on altered stress-related behavioral responses in an exploratory test and premature immunesenescence (Viveros et al., 2007; De la Fuente, 2010; Vida and De la Fuente, 2013).

Celiac disease (CD) is an immune-mediated enteropathy triggered by the intake of gluten proteins present in cereals such as wheat (gliadin and glutenin), barley (hordein) and rye (secalin) in genetically predisposed individuals. This disease is often manifested in early childhood after the first exposures to gluten with small intestinal villous atrophy and signs of malabsorption (Fasano and Catassi, 2005). Furthermore, an increasing number of adults are diagnosed with CD, suggesting that factors other than gluten exposure are involved.

CD results from the interaction between genetic, immunological and environmental factors (Nilsen et al., 1998; Fasano, 2001; Green and Cellier, 2007). Altered innate and adaptive immune responses to gluten proteins and an impairment of immunoregulatory mechanisms are involved in CD pathogenesis. Gluten proteins are rich in proline and glutamine residues, which make these proteins resistant to proteolytic degradation within the gastrointestinal tract (Silano et al., 2009). Moreover, the high content of glutamine residues makes gluten a good substrate for tissue transglutaminase activity, which deamidates glutamine into negatively charged glutamic acid with higher affinity for HLA-DQ2 and HLA-DQ8 molecules on antigen presenting cells (APCs) (Dubois and van Heel, 2008). APCs present these peptides to T cells, leading to an antigluten T-cell response, with a subsequent release of interferon (IFN)-γ and possibly interleukin (IL)-21, leading to epithelial damage (Kupfer and Jabri, 2012). Of the peptides identified as possible triggers for the adaptive immune response in CD patients, a 33-oligomer (57–89 fragment of α-gliadin) (referred as 33-mer) is considered to be especially pathogenic since it contains three of the most immunogenic epitopes (Anderson et al., 2000; Shan et al., 2002). Some gluten peptides also activate an aberrant innate immune response in epithelial and innate immune cells, contributing to the synthesis of pro-inflammatory cytokines (TNF-α, IL-15, etc.) and to CD pathogenesis (Cinova et al., 2007; Kupfer and Jabri, 2012).

CD is characterized by alterations in gut microbiota composition, as reported previously by our group (Nadal et al., 2007; Collado et al., 2009; Sanchez et al., 2012) and further confirmed by other authors (Wacklin et al., 2013). We have also provided evidence that specific components of the microbiota isolated from CD patients may influence immune responses of monocytes to gliadins (De Palma et al., 2010) as well as phenotypic and functional maturation of dendritic cells and their interactions with epithelial cells (De Palma et al., 2012). This could contribute to T-cell activation and disease progression as demonstrated *in vitro* and in ileal loop rat model (Cinova et al., 2011; De Palma et al., 2012). A recent study has suggested that the number of stressful events in life may be an additional factor favoring the clinical appearance of CD (Ciacci et al., 2013) and, likewise, stressful events are known to influence intestinal microbiota (Bailey et al., 2010, 2011; Powell et al., 2013).

Here, we hypothesize that dysfunctional nervous–immune communication due to altered stress-related behavior, associated with premature aging or due to chronological physiological aging, may contribute to a breakdown in gluten tolerance, thus explaining CD onset in late adulthood. To test this hypothesis we have assessed whether immunocompetent cells from mice with altered stress-related behavior and premature immune senescence and from chronologically aged mice respond differently to gliadin and intestinal bacteria stimulation (potential probiotics and pathogens), in terms of cytokine secretion and T cell population distribution, as compared to those of control mice.

2. Materials and methods

2.1. Animals, study design and experimental conditions

Adult (24 ± 1 weeks of age) and old (68 ± weeks of age) female outbred ICR-CD1 mice (*Mus musculus*) (Harlam Interfauna Ibérica, Spain) were classified as prematurely aged mice (PAM) and non-prematurely aged mice (NPAM) according to their different behavior in a T-maze, as previously described (De la Fuente et al., 1998; Viveros et al., 2001). This T-shaped maze essentially consists of three arms made of wood, whose internal surfaces are covered with black methacrylate. The inside dimensions of each arm are 10 cm wide, 25 cm long, and 10 cm high. The floor is made of 3 mm-thick cylindrical aluminum rods placed perpendicularly to the side walls. The test is performed by holding the mouse by the tip of its tail and placing it inside the "vertical" arm of the maze with its head facing the end wall. Its performance is evaluated with a chronometer to measure the time the animal takes to cross the intersection of the three arms with both hind legs. This test was performed four times, once a week, to sort the PAM (that required >10 s to complete exploration of the first arm at each test) from the NPAM (that completed the exploration in <10 s). Animals showing an intermediate response to the T-maze (26% of total population) were removed from the study. Thus, we had two groups of animals: one group including the NPAM population and another including the PAM population with NPAM/PAM ratios of 100:0 and 0:100, respectively. This test was always performed between 09:00 and 11:00 h under red light. Mice were housed in polyurethane boxes, at a constant temperature (22 ± 2 ° C) in sterile conditions inside an aseptic air negative-pressure environmental cabinet (Flufrance, Cachan, France), on a 12/12 h reversed light/dark cycle. All animals had access to tap water and standard Sander Mus pellets (A04 diet from Panlab L.S. Barcelona, Spain) *ad libitum*. This diet was in accordance with the recommendations of the American Institute of Nutrition for laboratory animals. The protocol was approved by the Experimental Animal Committee of the Complutense University of Madrid (Spain). Animals were treated according to the guidelines of the European Community Council Directives 1201/2005 EEC.

The animals were sacrificed by decapitation either at the age of 28 ± 1 weeks (Adult PAM and NPAM mice) or at the age of 72 ±1 weeks (Old PAM and NPAM mice). Therefore, there were four groups: adult PAM (AP), adult NPAM (ANP), old PAM (OP) and old NPAM (ONP). The spleen, mesenteric lymphoid nodes and intestine were removed immediately after the sacrifice.

2.2. Bacterial strains and culture conditions

The bacterial strains used were the following: *Bifidobacterium longum* CECT 7347, *Bifidobacterium bifidum* CECT 7365, *Escherichia coli* CBL2 and *Shigella* CBD8. *B. longum* CECT 7347and *B. bifidum* CECT 7365 were previously isolated from healthy human feces and identified by sequencing the 16S rRNA gene, as previously described (Medina et al., 2007; Izquierdo et al., 2008). *E. coli* CBL2 was isolated from symptom-free CD patients (under a gluten-free diet) and *Shigella* CBD8 was isolated from untreated CD patients and identified using the API20E system (BioMerieux, Lyon, France), as previously described (Sanchez et al., 2008). These strains had been evaluated as representatives of potentially pathogenic enteric bacteria and probiotic bacteria in previous studies related to CD (De Palma et al., 2010, 2012; Cinova et al., 2011).

Bifidobacteria were grown routinely in De Man, Rogosa and Sharpe (MRS) (ScharlauChemie SA, Barcelona, Spain) broth or agar with 0.05% cysteine and incubated at 37 °C under anaerobic conditions (AnaeroGen; Oxoid, Basingstoke, UK) for 22 h. Enterobacteria were routinely grown in Brain Hearth Broth and violet red bile dextrose (VRBD) agar (ScharlauChemie SA, Barcelona, Spain) at 37 ° C for 24 h under aerobic conditions.

For immunological assays, bacterial strains were grown in optimal condition and cells were harvested by centrifugation (6000 *g* for 15 min) at stationary growth phase, washed twice in phosphate-buffered saline (PBS) (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2) and re-suspended in PBS containing 20% glycerol. Aliquots of these suspensions were frozen in liquid nitrogen and stored at −80 °C until use. The number of live cells after storage was determined by