Food and Chemical Toxicology 88 (2016) 40–47

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Sodium benzoate, a food preservative, affects the functional and activation status of splenocytes at non cytotoxic dose

Ashish Yadav [a, b], Arvind Kumar [b], Mukul Das [a, **], Anurag Tripathi [a, *]

[a] Food Toxicology Lab, Food, Drug and Chemical Toxicology Group, CSIR- Indian Institute of Toxicology Research (CSIR-IITR), M.G. Marg, Lucknow, 226001, Uttar Pradesh, India

[b] Molecular Immunology Lab, School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi, 221005, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 3 July 2015
Received in revised form
11 December 2015
Accepted 14 December 2015
Available online 17 December 2015

Keywords:
Activation markers
Cytokines
Immunotoxicity
Lymphoproliferation
Splenocytes
Sodium benzoate

ABSTRACT

Sodium benzoate (SB) is a widely used food preservative due to its bacteriostatic and fungistatic properties. The acceptable daily intake of SB is 5 mg/kg-bw, however, it has been found to be used in the food commodities at relatively high levels (2119 mg/kg). Earlier studies on SB have shown its immunosuppressive properties, but comprehensive immunotoxicity data is lacking. Our studies have shown that SB was non cytotoxic in splenocytes up to 1000 μg/ml for 72 h, however at 2500 μg/ml it was found to be cytotoxic. Thus, 1000 μg/ml dose of SB was chosen for the subsequent experiments. SB significantly suppresses the proliferation of Con A and LPS stimulated splenocytes at 72 h, while allogenic response of T cells was significantly decreased after 96 h. SB did not affect the relative expression of CD3e or CD4 molecules following 72 h exposure, however, it downregulated the relative expression of CD8 co-receptor. Further, exposure of splenocytes to SB for 72 h led to reduced expression of CD28 and CD95, which play a vital role in T cell activation. SB also suppresses the relative expression of CD19, CD40 and CD95 receptors on B cells after 72 h. In addition to the functional responses, SB lowered the expression of IL4, IL6, IFNγ and IL17 cytokines in Con A stimulated splenocytes; and IL6, IFNγ and TNFα in LPS stimulated splenocytes following 48 h of exposure. Taken together, the present study is suggestive of the immunomodulatory potential of SB.

1. Introduction

Sodium benzoate (SB) is a well-known food preservative with bacteriostatic and fungistatic properties, which is widely used in various food preparations throughout the world. It is used in various food products such as fruit juices, pickles, salad dressings, fruit based fillings, jam and carbonated drinks. Joint FAO/WHO expert committee on food additives recommends acceptable daily intake levels of SB as 5 mg/kg-bw. However, recent survey suggests that it is being used at very high levels (2119 mg/kg) in the food articles exposing the population to health risks (Dixit et al., 2008). Consumption of SB beyond its ADI levels may produce toxic consequences in the exposed population. In a Swiss mice model it has been demonstrated that 4% levels of SB in diet result in death or growth depression within 5 weeks (Toth, 1984). Intake of SB increases liver weight and changes serum clinical parameters showing hepatotoxicity, however it did not affect the weight of heart, spleen and lungs (Fujitani, 1993).

SB has also been used as a therapeutic agent for various disease like urea cycle disorders, acute hepatic encephalopathy and multiple sclerosis (Brahmachari and Pahan, 2007; Sushma et al., 1992). It has been shown to ameliorate the clinical symptoms and disease progression of multiple sclerosis in the rodent model, however the study reveals that SB shifts the T helper cell response towards Th2 phenotype accompanied by enrichment of T-regulatory cells (Brahmachari and Pahan, 2007). However, unlike a disease model, enrichment of regulatory T-cells in healthy population may cause immunological abnormalities presaging an individual to contract diseases. SB has also been reported to interfere with cell cycle

Abbreviations: APC, Allophycocyanin; APC-Cy7, Allophycocyanin-Cyanine 7; CD, Clusters of Differentiation; Con A, Concanavalin A; DMEM, Dulbecco's Modified Eagles Medium; FITC, Fluorescein Isothiocyanate; IFN, Interferon; IL, Interleukin; LPS, Lipopolysaccharide; MLR, Mixed Lymphocyte Reaction; PE, Phycoerythrin; PerCP, Peridinin chlorophyll protein; Sodium Benzoate, SB; TNF, Tumor Necrosis Factor.

* Corresponding author.
** Corresponding author.
E-mail addresses: mditrc@rediffmail.com, mukul@iitr.res.in (M. Das), anurag1706@gmail.com, anuragtripathi@iitr.res.in (A. Tripathi).

http://dx.doi.org/10.1016/j.fct.2015.12.016
0278-6915/

progression in human lymphocytes leading to cytostaticity (Mpountoukas et al., 2008). It has been documented earlier that non cytotoxic concentrations of SB suppresses the cellular immune responses, however the comprehensive mechanism underlying the immunomodulatory effects of SB is not well understood (Maier et al., 2010).

The immune system comprises of various types of cells and an array of soluble mediators which are involved in the defense system of the organism (Klaassen, 2008). The functional assays of lymphocyte, analysis of cell surface receptor expression, cytokine levels and apoptosis etc. provide an indepth insight to understand the effect of xenobiotics on the immune system. Furthermore, USFDA Redbook guidelines and other immunotoxicity testing guidelines on food additives suggests a tiered approach comprising of "Level I", "Expanded level I" and "Level II" panel of assays for risk assessment of a xenobiotic compound (USEPA, 1996; Hinton, 2000; Gennari et al., 2005). The earlier studies indicating the immunomodulatory effects of SB emphasize on the need to carry out more elaborate immunotoxicity studies. In this study we therefore investigated the *in vitro* effects of SB on T-cell and B-cell responsiveness and further elaborated on its immunotoxicity mechanism by monitoring the SB induced changes on the expression level of cytokines, cell surface receptors and cell cycle distribution.

2. Material and methods

2.1. Chemicals and reagents

Sodium Benzoate (SB), Concanavalin A (Con A), lipopolysaccharide (LPS), mitomycin C, N-acetyl cysteine, non essential amino acids, MTT and most of the other reagents were purchased from Sigma Chemical Co (St. Luois, MO). Dulbecco's Modified Eagles Medium was purchased by Invitrogen Co (Carlsbad, CA) Cytometric Bead Assay Kit for $T_H1/T_H2/T_H17$ cytokines and Antibodies (Anti-CD3e-APC-Cy7/Alexafluor 488, Anti-CD4-FITC, Anti-CD8-PE, Anti-CD19-Alexafluor 700, Anti-CD25-APC, Anti-CD28-APC, Anti-CD40-APC, Anti-CD86-FITC and Anti-CD95-PE) for immunophenotyping were purchased from BD Biosciences (Sandiago, CA). Tritiated thymidine was purchased from Amersham Life science (Uppsala, Sweden).

2.2. Animals

Inbred strains of female Balb/c mice and Swiss mice (8–10 weeks old, 18–20 g) procured from the animal breeding colony of Indian Institute of Toxicology Research (Lucknow, India). Female animals were used in the present study because they posses strong immunologic response during the exposure to pathogens including enhanced antibody production upon immunication (Spencer et al., 1977; Goble and Konopka, 1973). Animals were acclimatized under standard laboratory condition for one week prior to the experiment and were housed in polycarbonate cage maintained at 22 ± 2 °C under standard laboratory conditions of light and dark cycles (12–12 h). Animals were kept on a normal diet and water ad libitum before sacrifice. Balb/c or Swiss mice were sacrificed according to the guidelines for the care and use of laboratory animals of Indian Institute of Toxicology Research, Lucknow, India.

2.3. Splenocyte culture

Splenocytes were isolated and cultured as described earlier with some modifications (Kruisbeek, 2001). Spleens were washed with cold phosphate buffered saline and cell suspension was prepared by mincing tissue in incomplete Dulbecco's modified eagles medium (DMEM). Erythrocytes were lysed with erythrocytes lysis buffer (150 mM NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4). The cells were then washed two times with incomplete medium and centrifuged (300 × g). Cells were resuspended in DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, 25 mM dextrose and 50 μM β-mercaptoethanol. The cells were cultured at a concentration of 2×10^6 cells/ml and incubated overnight for acclimatization.

2.4. Cytotoxicity assays

Cytotoxicity of SB in splenocytes was determined by MTT assay as described earlier (Mosmann, 1983). For MTT assay, cells were cultured and treated with different concentrations of SB (0–2500 μg/ml) for 72 h 4 h prior to the termination of experiment, MTT was added to cell culture at 0.5 mg/ml concentration. At the end of the experiment, culture plate were centrifuged and cell pellet was dissolved in DMSO and further read in a plate reader (BioTek Instruments Inc, Vermont, USA) at 550 nm and 660 nm.

2.5. Lymphoproliferation assays

Concanavalin A (Con A) and Lipopolysaccharides (LPS) were used to induce proliferation in T cell and B cell populations present in splenocytes culture, respectively. Cultured splenocytes were treated with Con A (5 μg/ml) or LPS (10 μg/ml) in presence of SB and incubated at 37 °C for 72 h. The relative fold proliferation of cells was determined by the uptake of tritiated thymidine in cells. Tritiated thymidine (2 μ Ci/ml) was added to the cultures 18 h prior to the end point. The cells were then harvested with cell harvestor (Perkin Elmer) in glass fiber filters. These filters were transferred to scintillation cocktail-W (Sisco Research Laboratories Pvt. Ltd. Mumbai, India), and β-counts were recorded on β-counter (Hewlett–Packard, Palo Alto, CA).

2.6. Cell cycle phase distribution

SB treated splenocyte were washed and fixed in 70% ethanol for 2 h at −20 °C. Fixed cells (1×10^6 cells) were then washed with PBS and stained with PI solution (2 mg DNase-free RNase A and 200 μl of 1 mg/ml propidium iodide in 10 ml of 0.1% (v/v) Triton X-100/PBS) for 30 min and analyzed by flow cytometry.

2.7. Mixed lymphocyte reaction

For mixed lymphocyte reaction (MLR), stimulator cells (Swiss albino splenocytes) (2×10^7 cells/ml) were treated with mitomycin C (25 μg/ml) for 30 min to prevent mitogenic activity. Responder cells (Balb/c splenocytes without mitomycin C treatment) were co-cultured with stimulator cells in a ratio of 1:4 (0.5×10^5: 2×10^5 cells per 200 μl). For MLR, the splenocyte culture media was additionally supplemented with 1% nonessential amino acids and 10 mM N-acetyl cysteine. The co-culture was incubated at 37 °C in a humidified CO_2 incubator in the presence or absence of SB for 5 days. The relative fold proliferation of cells was determined by the uptake of tritiated thymidine in responder cell.

2.8. Immunophenotyping

Cells were labeled with surface marker specific antibodies for the identification of individual populations of B cells and T cells in control and SB treated groups after 72 h of treatment. The cells were suspended in staining buffer (2% FBS, 1% sodium azide in PBS) and stained with Anti-CD3e-APC-Cy7, Anti-CD4-FITC, Anti-CD8-APC, Anti-CD19-Alexafluor700, Anti-CD25-APC, Anti-CD28-APC,