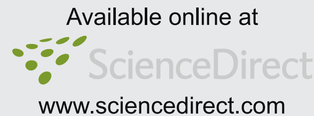
Biomedicine & Aging Pathology 1 (2011) 61–64



Original article

A comparative analysis of immunomodulatory potential of Seabuckthorn leaf extract in young and old mice

K.P. Mishra*, R. Mishra, A.P. Yadav, B. Jayashankar, S. Chanda, L. Ganju

Immunomodulation Laboratory, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi 110054, India

ARTICLE INFO

Article history:
Received 12 August 2010
Accepted 20 February 2011
Available online 6 May 2011

Keywords:
Seabuckthorn
Aging
Splenocytes
MHC-II
CD25

ABSTRACT

Aging is associated with increased susceptibility to infection, cancer or autoimmune diseases. One strategy to combat the increased morbidity and mortality related to these diseases in the elderly is to identify ways to prevent the immunological deficiencies. The immune system of the elderly may be boosted by use of immunomodulators. To determine the effect of aging on splenocyte proliferation, IFN-γ, CD 25 and MHC class II antigen expression, we have compared the immune response of young (3 month) and old (> 17 month) group of mice immunized with diphtheria toxoid (DT) in combination with complete freund's adjuvant CFA) or Seabucthorn leaf alcoholic extract (SBTLAE). The data observed in the present study indicates that the IFN-γ production, CD25 and MHC-II expression is lowered in elderly mice however use of SBTLAE increased IFN-γ, CD25 and MHC II expression. The splenocyte proliferation was not very much affected in comparison to young group of mice. The present study suggests that Seabuckthorn may be used as immunomodulators to strengthen the immune response of aged ones.

© 2011 Elsevier Masson SAS. All rights reserved.

1. Introduction

Aging is a complex process that negatively impacts the development of the immune system and its ability to function. The mechanisms that underlie these age-related defects are broad and range from defects in the haematopoietic bone marrow to defects in peripheral lymphocyte migration, maturation and function [1]. In Western countries, the mortality rate increases 25 times more rapidly in individuals over 60 years old compared to people aged 25–44. Causes of death in aged people are increased compared with individuals between 25 and 44 years old, cancer 43-fold, pneumonia and influenza 89-fold, heart disease 92-fold and stroke and chronic lung disease greater than 100-fold [2]. Thus far, to understand aging mechanisms, much attention has been paid to gene mutations in invertebrates and caloric restriction in rodents. However, these data suggest a key role for immunity in the survival of the elderly because susceptibility to these diseases depends at least in part on optimal immune function [3,4]. So, a better understanding of the aging immune system may provide the most important clues for slowing the inevitable decline associated with the passage of time.

The modulation of the immune system in an individual is achieved by introducing the immunomodulatory agents, which can enhance the immunological responsiveness of an organism by interfering with its regulatory mechanism. The immunomodulator used in the present study was Seabuckthorn (*Hippophae rhamnoides*), which belongs to family Eleagnaceae. *Hippophae* is usually found at an altitude of 2000–4500 m in cold climates, though it can grow at both higher and lower altitudes, in sandy soils. Seabuckthorn is a deciduous shrub with yellow orange fruit, which is known as medicinal plant of Himalayan region. Its leaves as well as fruits are of great importance in wide research area [5,6].

The immunomodulatory potential of Seabuckthorn leaf extract is well documented [5–8] however the immunological consequences in different age group subjects are still unknown. In the present study, we have compared the immunomodulatory potential of Seabucthorn leaf alcoholic extract (SBTLAE) in young and old mice. The data suggest that in elderly mice the immune response was weaker in comparison to younger group of mice. However the immune response of elderly mice significantly enhanced using SBTLAE, which may protect the elderly subject from different opportunistic infections.

2. Materials and methods

2.1. Reagents required

RPMI-1640 media, Fetal bovine serum (FBS), Concanavalin A (Con A), Tween-20, Complete Freund's Adjutants (CFA), Incomplete Freund's Adjuvant (IFA) and MTT were obtained from sigma, USA. Ammonium chloride (NH_4Cl), potassium chloride (KCl), sodium chloride (NaCl), sodium corbonate (Na_2CO_3) sodium bicarbonate

* Corresponding author. Tel.: +23883163; fax: +23932869.
E-mail address: kpmpgi@rediffmail.com (K.P. Mishra).

2210-5220/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biomag.2011.02.001

potassium di hydrogen phosphate (KH_2PO_4), di sodium hydrogen phosphate (Na_2HPO_4) and di methyl sulphoxide (DMSO) and ethyl alcohol were obtained from Qualigens fine chemicals Mumbai, India. Anti mouse MHC-II-Phycoerythrin (PE), anti mouse CD25-PE conjugated antibodies and Mouse IL-4 and IFN-γ ELISA kits were obtained from BD-Pharmingen. USA tetramethylbenzidine (TMB), hydrogen peroxide (H_2O_2), sulfuric acid (H_2SO_4), was obtained from Biotrand, Germany. Antigen-DT (diphtheria toxoid) was obtained from CRI, Casuali, India.

2.2. Plant extract

The leaves of Seabuckthorn plant were collected from hilly region of Western Himalayas, India, and dried under shade. The dried leaves were powdered and extracted with 70% ethanol overnight. The supernatant was saved and the residue was re-extracted with 70% ethanol. The process was repeated four times for complete extraction. The supernatant of leaf extracts were pooled and dried under vacuum at 50 °C. For experimental use the dried extract was dissolved in 70% ethyl alcohol. The extract was further diluted in RPMI medium according to the requirement for various assays.

2.3. Animals

The young Balb/c mice of 2–4 months age, 30 g weight and old Balb/c mice of greater than 17 months age, 40 g weight were used in the present study.

2.4. Grouping of animals

Young and old group of mice were divided into four groups of each namely:

- untreated;
- DT + CFA;
- DT + SBTLAE;
- SBTLAE.

2.5. Immunization schedule

Five μg of DT in CFA or SBTLAE (100 mg/kg body wt) and SBTLAE (100 mg/kg body wt) alone were administered i.p. On day '0'. 1st booster dose was given on day 14th with IFA. Animals were bled and serum was separated after 21 days of immunization.

2.6. Isolation of splenocytes

Young and old mice from different groups were anesthetized with Die ethyl ether. Spleens were removed aseptically post-mortem, cut and passed through a wire mesh to obtain a single cell suspension. Furthermore, cells were centrifuged at 1500 rpm for 10 min and pellet was treated with 0.84% ammonium chloride to lyse red blood cells (RBC). After RBC lysis cells were washed twice with PBS. For lymphocyte proliferation/cell viability 1×10^6 cell/mL were cultured in RPMI supplemented with 10% FCS [9].

2.7. Splenocyte proliferation

Mitochondrial respiration, an indicator of cell viability, was assessed by mitochondria-dependent reduction of a yellow tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) to insoluble purple formazan by dehydrogenase [9]. MTT Proliferation assay was performed to obtain qualitative information about the viability of mononuclear cells. The absorbance value is directly proportional to the number of viable cells in the samples.

Splenocytes were seeded at a concentration of 1×10^6 cells/mL in 96-well plate with or without Con-A (5 μg/mL). After incubation for 72 h, 20 μL of MTT stock solution (5 mg/mL) was added to each well and the plates were further incubated for 4 h at 37 °C in CO_2 incubator. The supernatant was removed and 100 μL of DMSO was added to each well to solubilize water insoluble purple formazan crystals. The absorbance at 570 nm was measured using ELISA reader.

2.8. IFN-γ production and estimation

IFN-γ was estimated by sandwich ELISA method in serum of different groups of young and old mice as per instruction manual from BD-Pharmingen (USA). Estimation of IFN-γ in serum samples was carried out as solid phase enzyme immunoassay with ELISA kit (BD-Pharmingen USA) based on multiple antibody sandwich principle [10]. The ELISA plate was coated with anti-IFN-γ to capture the specific cytokine present in the standard and serum samples. The detection antibody conjugated to biotin was added to the well followed by streptavidine/HRP incubation for 20 min. At the end color was developed using peroxide/TMB substrate solution. The substrate initiated a peroxidase catalyzed color change, which was stopped within 15 min by acidification with stop solution. Absorbance was measured by ELISA reader (Biotek, USA) at 450 nm. The absorbance measured at 450 nm was proportional to the concentration of IFN-γ in the samples. Standard curve was obtained by plotting known concentrations of IFN-γ versus absorbance. The cytokine concentrations in experimental samples were determined against standard curve. Results are presented as concentrations in pg/mL.

2.9. Flowcytometry for surface expression

Cell surface expression of CD-25 and MHC-II were evaluated in splenocytes of young and old group of mice by PE conjugated monoclonal antibody using flow cytometer. Further, it was incubated with antibodies at room temperature in the dark for 30 minutes. Cells were then washed twice with PBS (pH 7.2) and finally resuspended in 500 μL of PBS. Background fluorescence was assessed with the appropriate isotype and flourochrome matched control monoclonal antibody. Twenty thousand events were analyzed in FACScaliber using Cell Quest software [7].

2.10. Statistical analysis

The data were analyzed using SPSS-10 software. All the data is presented as Mean ± SD (standard deviation). Analysis of variance and Student's *t* test was used for statistical analysis. The *P* value less than 0.05 was considered as level of significance.

3. Results

3.1. Splenocyte proliferative response against Con-A

The splenocyte prolifation response was performed after 21 days of immunization. The splenocytes of different groups of young and old mice were treated with or without Con-A for 72 h. The reduction of MTT was read at 570 nm. The Stimulation index (SI) was calculated by dividing mean OD of Con-A treated cells with untreated control. No significant alterations were found in the splenocyte proliferation of young and old mice (Fig. 1).

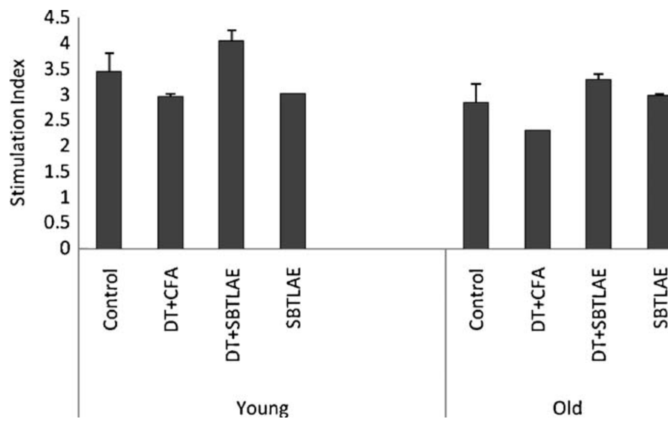


Fig. 1. Young and old group of mice splenocytes (21 days after immunization) were treated with or without Con-A for 72 h and stained with MTT dye. OD Read at 570 nm. Data represented as Mean ± SD.

3.2. IFN-γ estimation

IFN-γ was measured in young and old group of mice to compare the Immunomodulatory potential of SBTLAE. The level of IFN-γ was significantly higher in all-young groups of mice in comparison to old group of mice (Fig. 2). The use of immunomodulator could not change the production of IFN-γ in older groups of mice however the SBTLAE significantly enhanced the production of IFN-γ in young groups. The level of IFN-γ was quite low in older groups of mice hence here it is also established that aging is significantly affecting the production of IFN-γ level.

3.3. CD 25 expression

The CD 25 expression was significantly lowered in old group of mice immunized with DT in comparison to young group of mice. The lower expression of CD25 (IL-2 receptor alpha chain) suggests the decreased immune responsiveness of the T cells in elderly mice. Dot plot analysis of the lymphocyte population in unimmunized (control) young groups of mice showed 51.5% and old group of mice showed 50% while DT and CFA immunized young group of mice showed 68% cells stained with anti-CD25 antibody while old group showed 55% of CD25 positive cells. Furthermore, DT and SBTLAE immunized young group of mice showed 59% of CD25 expression while old group showed 54% of CD25 positive cells. SBTLAE alone immunized young and old gruoup of mice showed 61 and 66% of CD25 positive cells (Fig. 3).

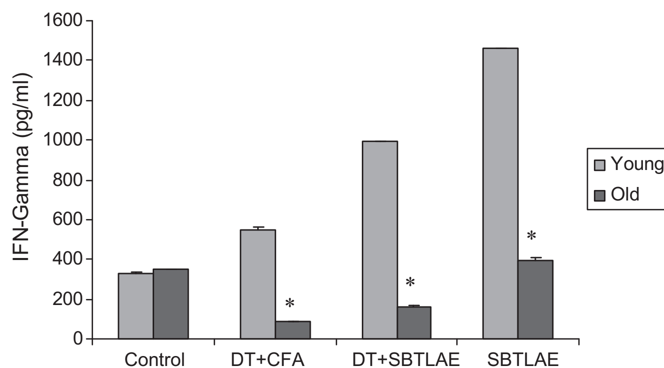


Fig. 2. Determination of IFN-γ level in sera of different groups of young and old mice after 21 days of immunization. Data represented as Mean ± SD. $^{*}P < 0.01$ vs. respective young group.

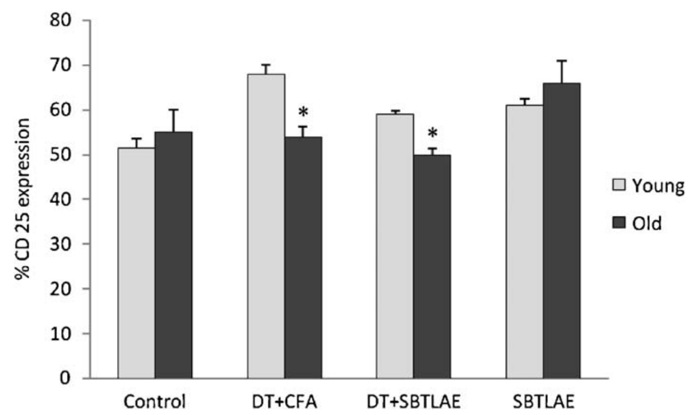


Fig. 3. Comparative analysis of CD25 expression in young and old Balb/c mice. Five μg of DT in CFA or SBTLAE (100 mg/kg body wt) and SBTLAE (100 mg/kg body wt) alone were administered i.p. On day '0'. 1st booster dose was given on day 14th with IFA. Animals were sacrificed after 21 days of immunization and CD25 expression on splenocytes were evaluated by flowcytometry. $^{*}P < 0.05$ vs. respective young group. Data represented as Mean ± SD.

3.4. MHC-II expression

MHC-II expression was significantly lowered in old group of mice immunized with DT in comparison to young group of mice. The lower expression of MHC-II suggests the decreased immune responsiveness of the T cells in elderly mice. Dot plot analysis of the lymphocyte population in DT and CFA immunized young group of mice showed 46% cells stained with anti-MHC-II antibody while old group showed 34% of MHC-II positive cells. DT and SBTLAE immunized young group of mice showed 37% of MHC-II expression while old group showed 26% of MHC-II positive cells. SBTLAE alone immunized young and old group of mice showed 24 and 23% of MHC-II positive cells (Fig. 4).

4. Discussion

Understanding the immune response of the elderly is becoming an urgent health care priority with the projected rapid expansion of this population. It is well known that numerous immunological deficiencies emerge with age, resulting in increased susceptibility of the elderly to infection, cancer or autoimmune diseases. One strategy to combat the increased morbidity and mortality associated with infection, cancer or autoimmunity in the elderly is

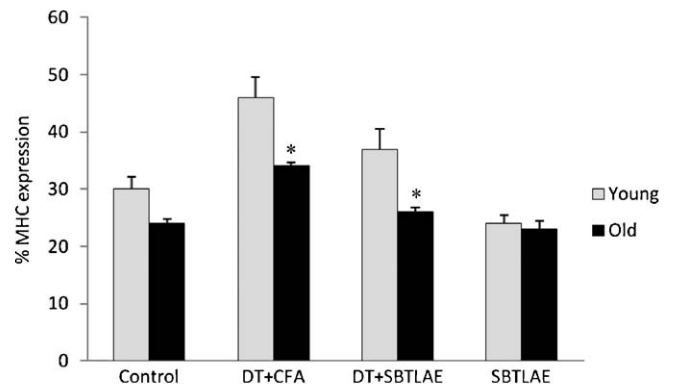


Fig. 4. Comparative analysis of MHC-II expression in young and old Balb/c mice. Five μg of DT in CFA or SBTLAE (100 mg/kg body wt) and SBTLAE (100 mg/kg body wt) alone were administered i.p. On day '0'. 1st booster dose was given on day 14th with IFA. Animals were sacrificed after 21 days of immunization and MHC-II expression on splenocytes were evaluated by flowcytometry. $^{*}P < 0.05$ vs. respective young group. Data represented as Mean ± SD.

to identify ways to prevent the immunological deficiencies [3,4]. In the present study we have used an immunomodulator SBT-LAE and compared the immune response of young and old mice by looking at splenocyte proliferation, IFN-γ secretion, CD 25 and MHC-II expression. The data observed in the present study indicated that the IFN-γ production, CD 25 and MHC-II expression is lowered in elderly mice though the splenocyte proliferation was not very much affected in comparison to young group of mice.

MHC class II proteins play a key role in the development and maintenance of the immune system [11]. They participate in the generation of the T-cell repertoire in the thymus and are required for antigen presentation to T lymphocytes [12]. Aberrant expression of class II proteins has been linked to immune dysfunction. Lack of class II expression in humans [13] and in animal models [14] leads to severe combined immunodeficiency and abnormal expression is probably linked to the development of autoimmune diseases [15]. The level of class II antigen expression has been correlated with the intensity of the immune response in physiological conditions [16]. Class II proteins are normally expressed on a limited number of cell types, including B, thymic epithelial, dendritic, and glial cells, as well as activated macrophages [17]. Our data also suggest that in elderly mice the level of MHC-II expression was lowered in comparison to young group of mice. The MHC II expression is also regulated by cytokines, mainly IFN-γ [18], primarily through transcriptional activation. The decreased level of MHC-II expression in old mice could be because of decreased level of IFN-γ. The advancing age is associated with significant alternations in the function of mouse T cells [19]. Aging leads to a decrease in the ability to mount strong T responses to new antigens and to previously encountered recall antigens in mice [1]. In the present study, the level of IFN-γ in aged mice was significantly decreased as compared to young mice. However the level of IFN-γ was markedly increased in the elderly mice treated with SBTLAE. CD25 is a unique subunit of the high affinity IL-2 receptor (IL-2Rα). T cell activation in young and old mice was evaluated. It was observed that IL-2 receptor alpha chain expression was significantly lowered in comparison to young group of mice. So the immunomodulator used in the present study could be useful for combating the age related dysfunctioning of the immune system.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

References

[1] Miller RA. The aging immune system: primer and prospectus. Science 1996;273:70–4.
[2] Troen BR. The biology of aging. Mt Sinai J Med 2003;70:3–22.
[3] Pawelec G, Barnett Y, Forsey R, Frasca D, Globerson A, McLeod J, et al. T cells and aging. Front Biosci 2002;7:d1056–183.
[4] Candore G, Colonna-Romano G, Lio D, Caruso C. Immunological and immunogenetic markers of successful and unsuccessful ageing. Advances in Cell Aging and Gerontology 2003;13:29–45.
[5] Ganju L, Padwad Y, Singh R, Karan D, Chanda S, Chopra MK, et al. Anti-inflammatory activity of Seabuckthorn (*Hippophae rhamnoides*) leaves. Int Immunopharmacol 2005;5:1675–84.
[6] Padwad Y, Ganju LRC. Effects of leaf extract of Seabuckthorn on lipopolysacchride induced inflammatory response in murine macrophages. Int Immunopharmacology 2006;6:46–52.
[7] Mishra KP, Chanda S, Dev Karan, Ganju L, Sawhney RC. Effect of seabuckthorn flavon on immune system: an in-vitro approach. Phytotherapy Research 2008;22:1490–5.
[8] Mishra KP, Ganju L, Chanda S, Dev Karan, Sawhney RC. Aqueous extract of Rhodiola imbricata rhizome stimulates Toll Like Receptor 4, Granzyme B and Th1 cytokines in-*vitro*. Immunobiology 2009;214(1):27–31.
[9] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55.
[10] Mishra KP, Singh VK, Rani R, Yadav VS, Chandran V, Srivastava SP, et al. Effect of lead exposure on the immune response of some occupationally exposed individuals. Toxicology 2003;188:251–9.
[11] Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994;76:287–99.
[12] von Boehmer H. Positive selection of lymphocytes. Cell 1994;76:219–28.
[13] Mach B, Steimle V, Martinez-Soria E, Reith W. Regulation of MHC class II genes: lessons from a disease. Annu Rev Immunol 1996;14:301–31.
[14] Cosgrove D, Gray D, Dierich A. Mice lacking MHC class II molecules. Cell 1991;66:1051–66.
[15] Bottazzo GF, Pujol-Borrell R, Hanafusa T, Feldmann M. Role of aberrant HLA-DR expression and antigen presentation in induction of endocrine autoimmunity. Lancet 1983;2:1115–9.
[16] Otten LA, Steimle V, Bontron S, Mach B. Quantitative control of MHC expression by the transactivator CIITA. Eur J Immunol 1998;28:473–8.
[17] Glimcher LH, Kara CJ. Sequences and factors: a guide to MHC class-II transcription. Annu Rev Immunol 1992;10:13–49.
[18] King DP, Jones PP. Induction of Ia and H-2 antigens on a macrophage cell line by immune interferon. J Immunol 1983;13:315–8.
[19] Miller RA. Aging immune function. Int Rev Cytol 1991;124:187–215.