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RESEARCH ARTICLE

The effect of MS14 on innate and cellular immune responses in BALB/c mice

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Abstract

MS14 is an Iranian natural preparation of herbal—marine source with no obvious toxicity in oral administration, which possesses anti-inflammatory and immunomodulatory effects. In this study, the effect of oral administration of MS14 on nitric oxide (NO) production of peritoneal macrophages and lymphocyte Th1 cytokines and delayed-type hypersensitivity (DTH) test in BALB/c mice were investigated. Peritoneal macrophages were cultured and NO production was measured by Griess method. Viability of macrophages was assayed by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) test. Interleukin-2 (IL-2) and INF γ levels in supernatant of spleen lymphocytes culture were assayed by enzyme-linked immunosorbent assay kit. For DTH test the mice were immunized with sheep red blood cell and DTH was measured 24 h after the last immunization of mice. NO production of macrophages has been diminished significantly in MS14 treated group (about 40%) at the presence or absence of stimulators. Macrophage viability had no significant alteration after MS14 administration. However, interferon- γ production of lymphocytes was significantly decreased in MS14 group both at the absence or presence of concanavalin A (ConA; about 50%); IL-2 production declined about 20% at the presence of ConA. In comparison with the control group, MS14 had no statistically significant effect on DTH test. The results have pointed that MS14 may have immunomodulatory potentials at least through its decreasing effect on NO production of macrophages and level of Th1 cytokine pattern.

Keywords: MS14, immunomodulator; nitric oxide, interferon- γ , interleukin-2, delayed-type hypersensitivity test

Introduction

Innate and acquired immunity are two important arms of immune system. Innate immunity is rapid, relatively non-specific, and does not result in immunologic memory, whereas acquired immunity involves antibodies and T-cells, which can recognize the specific foreign agents and invoke long-term memory.⁽¹⁾ Innate immunity in which macrophages are the key components is the prominent immunity in many living beings.^(2,3) The macrophages are also the important components of inflammatory reactions producing various cytokines and metabolites including nitric oxide (NO).⁽⁴⁾

In acquired immunity, T-cell responses are traditionally divided into two main subpopulations, Th1 and Th2, according to differences in their cytokine expression profiles which their importance in many diseases are known.⁽⁵⁾ INF- γ and interleukin-2 (IL-2) are among the major cytokines produced by Th1 cells; contributing to cell-mediated inflammatory immune responses, while IL-4 and IL-10 are secreted by Th2 cells, mediate various other responses including the humoral responses.⁽⁶⁾ The imbalance of Th1/Th2 status may cause infection, autoimmune disorders, and allergic diseases.⁽⁵⁾ Moreover, delayed-type hypersensitivity (DTH) reactions are antigen-specific,

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cell-mediated immune responses which depending on the antigen involved, mediate beneficial (resistance to viruses, bacteria, fungi, and tumors) or harmful (allergic dermatitis, autoimmunity) aspects of immune function.^(7,8) Therefore, DTH and IL-2 and INF γ may reflect the situation of cellular immune response; abnormal cytokine production has been demonstrated in many pathological states such as autoimmune, allergic, etc.⁽⁹⁾ On the other hand, immunomodulators are very important in clinical medicine, because they manipulate immune responses and can regulate the cytokines production.⁽¹⁰⁾ Nowadays many researchers do ample efforts in order to find natural compounds—especially from herbal origin—with the ability of modulating immune responses (immunomodulators). Most of the clinical effects of these plants have been reportedly attributable to its immunoregulatory activity.⁽¹¹⁾ MS14 is a herbal-marine component that contains 90% *Penaeus laticulatus* (king prawn), 5% *Apium graveolens* and 5% *Hypericum perforatum*.⁽¹²⁾ This preparation, made by Iranian traditional medicine scientists, has no toxicity when fed even in very high doses.⁽¹³⁾ It has been used for the treatment of multiple sclerosis (MS) patients and animal model (experimental autoimmune encephalitis).^(12,14) In order to investigate more details about anti-inflammatory effects of MS14 and its effects on cellular immunity the effects of orally administered MS14 on macrophage responses including macrophages viability and [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT)] and their NO production were evaluated in mice, in this study. Th1 cytokines (IL-2 and INF γ) and DTH reaction were also assessed in order to evaluate *in vivo* cellular immune response.

Materials and methods

Mice and MS14

Inbred female BALB/c mice (6–8 weeks old) maintained under pathogen-free conditions were used throughout the study. These animals were randomly selected and divided into control and drug groups (five mice per group).

MS14 powder was produced by Department of Pharmacology, Shahed University, Tehran, Iran.⁽¹²⁾ The powder was dissolved in sterile normal saline and 100 μ l were administered orally using feeding tube (according to test 50, 100, 200 mg/kg for 5 or 6 days). The mice in control group was administered the same volume of normal saline orally.

Macrophage cell isolation and culture

Twenty-four hours after last administration, the mice were anesthetized and the skin of chest and abdomen of mice was carefully dissected without opening the peritoneum. Peritoneal exudate cells were obtained from each mouse using lavage method in which 5 ml of cold normal saline was twice injected intraperitoneally. The abdomen was massaged and 90–95% of the injected volume was recovered.⁽¹⁵⁾ The cells were centrifuged, washed, and

resuspended in Roswell Park Memorial Institute medium 1640 (RPMI1640; Gibco) medium supplemented with 10% fetal calf serum (Gibco) and were counted using trypan blue dye to detect dead cells which were less than 3% all the times. Then 4×10^5 cell/well were cultured in 96-well microplates (Falcon) and incubated at 37°C and 5% CO₂ for 2h. The non-adherent cells were removed by washing the plate with normal saline (37°C) and the adherent cells were incubated for 24h with or without stimulators (fMLP (50 μ g/ml), PMA (25 ng/ml), and LPS (10 μ g/ml) according to test. All procedures were conducted under aseptic condition and the normal saline was sterile and LPS free (injectable grade).

Preparation of splenic lymphocytes

Spleen of mice was separated and the single cell suspension was prepared. Then, red blood cells were lysed by suspending cells in 0.83% NH₄Cl in 10%Tris-HCl buffer followed by fetal bovine serum (FBS) increasing. The cell suspensions were centrifuged (3000 rpm for 10 min) and resuspended gently in RPMI1640 + 10% FBS. The splenic cells were counted and cultured in 96-well flat-bottomed microtiter plate in 2×10^5 cell/well. Concanavalin A (ConA) mitogen (25 μ g/ml final concentrations) was added to half of samples and the plates were incubated. Finally, the supernatants were collected after 48h and stored at -70°C for cytokine assay.⁽¹⁶⁾

NO production of peritoneal macrophages

NO production of peritoneal macrophages was assayed by measuring nitrite in supernatant of cultured macrophages (Griess method) after 18h. Briefly, 50 μ l of supernatant from each well of microplate was transferred to a 96-well flat-bottom microtiter plate and 50 μ l of 1% sulfanilamide (Fluka) solution and 50 μ l of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Merck) solution (both in 5% phosphoric acid) were added to each sample and all standards. The absorbencies were read at 492 nm and the amount of nitrite was calculated as μ M.

MTT test

A 5 mg/ml solution of MTT (Merck) was prepared in phosphate buffered saline (PBS), filtered and stored at -20°C. When needed, the solution was thawed and added to each well as one-tenth of its volume. Then the cells were incubated at 37°C and 5% CO₂ for 4h. The supernatants were gently removed and the formazan crystals were resolved in acidic isopropanol (0.04 M HCl in isopropanol) and absorbance was read at 492 nm.

Measurement of cytokines by ELISA

Culture supernatants from lymphocyte were examined for Th1 cytokines namely, IL-2 and INF γ , using enzyme-linked immunosorbent assay (ELISA) kits from Biosource (Switzerland). According to manufacturer's instructions, briefly flat-bottom 96-well plates were coated overnight at 4°C with coating IL-2 or interferon- γ (IFN γ) mAbs. The primary mAbs were discarded and the plates were

blocked with blocking buffer for 1 h at room temperature. Then, the plates were washed three times with wash buffer (0.05% Tween 20 in PBS) and blotted on a paper towel. Diluted standards and samples were added in duplicate and the plates were incubated for overnight at room temperature. The supernatant was discarded and the wells were washed three times with wash buffer. IL-2 or IFN γ detecting second antibody was added and incubated for 30 min at room temperature. After washing, tetramethyl benzidine substrate solution was added. The color was allowed to develop for 30 min in the dark place before the reaction was quenched with a stop solution (1.8 N H $_2$ SO $_4$). The plates were read at 450 nm and the sample concentrations were determined using a standard curve.

Generation of DTH response to sheep red blood cell

At first day of trial, 3% sheep red blood cell (SRBC) suspension (10 8 SRBC in 0.1 ml normal saline) subcutaneously (s.c.) injected at the base of the tail in a 0.1 ml volume. The DTH reaction was elicited after 5 days via s.c. administration of 3% SRBC suspension normal saline (0.1 ml volume) into the one-hind footpad. Then, the footpad swelling was measured with a caliper (accuracy 0.05 mm) 24 h later.⁽¹⁷⁾

The antigen-specific reaction to SRBC was calculated by subtracting SRBC-elicited reaction in non-sensitized footpad of mice from SRBC-elicited reaction of sensitized footpad. According to this frame⁽¹⁸⁾:

$$\frac{\text{Diameter of injection footpad} - \text{Diameter of non-injection footpad}}{\text{Diameter of non-injection footpad}}$$

Statistical analysis

Data are presented as mean \pm SEM. Comparisons between the control and drug groups were performed with Students *t*-test and for more than two groups, analysis of variance test was down.

Results

The effect of various doses and time periods of MS14 administration on NO production of peritoneal macrophages

As shown in Table 1 the NO production of peritoneal macrophages was decreased significantly when the MS14 at doses of 100 or 200 mg/kg has been administered for 6 days.

Table 1. The effect of MS14 on NO production of macrophages.

Administration period	Control	MS14 received groups		
		50 (mg/ml)	100 (mg/ml)	200 (mg/ml)
6 Days	107.22 \pm 4.77 μ M	100.77 \pm 3.95 μ M	86.729 \pm 1.15 μ M*	85.88 \pm 1.01 μ M*

The effect of oral administration of various doses of MS14 (50, 100, 200 mg/kg) on the nitric oxide (NO) production of mouse peritoneal macrophages. The macrophages were cultured at 2×10^5 and the NO production was assayed by Griess method. All data are represented as means \pm SEM.

*Denoted significant differences with control group $P < 0.01$.

No significant alteration has been observed when 50 mg/kg has been used.

The Effect of MS14 (100 mg/kg) on NO production of stimulated macrophages

NO production of stimulated (PMA + fMLP or PMA + LPS) and non-stimulated peritoneal macrophages were evaluated after exposure to 100 mg/kg of MS14. As shown in Figure 1, both stimulating conditions significantly augmented NO production of peritoneal macrophages in control group ($P < 0.05$). In MS14 administered group, the observed decrease was about 65% in non-stimulated and 77% in stimulated macrophages with $P < 0.005$, $P < 0.0003$, and $P < 0.002$, respectively.

The Effect of MS14 (100 mg/kg) on viability of peritoneal macrophage (MTT test)

The stimulators did not significantly increase the vital activity of macrophages (MTT test). Administration of MS14 for 5 days has not affect viability of non-stimulated macrophages, but at the presence of stimulators (fMLP + LPS), reduced viability of macrophages was observed in MS14 group (Table 2).

The Effect of MS14 (100 mg/kg) on IL-2 and IFN γ spleen lymphocyte

Mice splenocytes from mice in control and MS14 treated (for 5 days) groups were cultured and ConA were added to half of cultures in each group then incubated for 48 h, the supernatant was collected and IL-2 was measured. IL-2 did not show any significant alteration in 100 mg/kg MS14 treated mice, but as Figure 2 shows, IL-2 production by lymphocyte significantly decrease at the presence of ConA, from 915.53 ± 26.324 to 732.91 ± 50.109 ($P < 0.003$).

For measurement of IFN γ , splenocytes from control and MS14 treated groups were cultured in RPMI1640 with 10% FBS and ConA were added to half of cultures in each group and supernatant was collected after 48 h incubation., result shown that 100 mg/kg of MS14 for 5 days administer, significantly decrease IFN γ production of lymphocyte from 81.71 ± 19.02 to 39.18 ± 5.82 ($P < 0.04$), the same result obtains at the presence of ConA, IFN γ level in control group was 10179.8 ± 78.69 and in MS14 group was 9788.13 ± 13 ($P < 0.01$, Figure 3).

The Effect of MS14 on DTH test

Control and MS14 treated groups of BALB/c mice, immunized s.c. at the base of the tail with 10 8 SRBC. The DTH reaction was determined by measurement swelling of

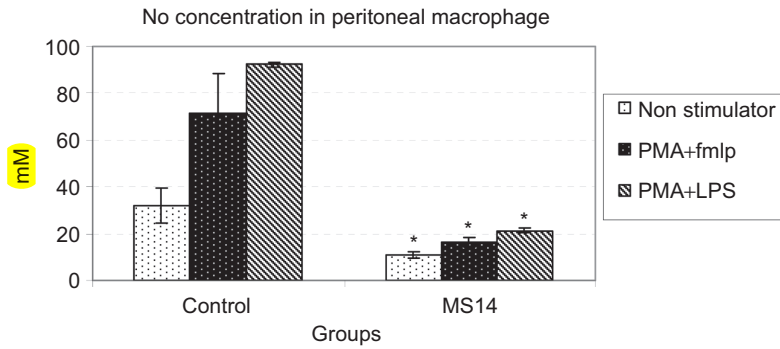


Figure 1. The effect of 5 days oral administration of MS14 (100 mg/kg) on the nitric oxide (NO) production of mouse peritoneal macrophages. The macrophages were cultured at 2×10^5 and after 18 h the NO production was assayed by Griess method. All data are represented as means \pm SEM. * $P < 0.01$ denoted significant differences with control group.

Table 2. The effect of MS14 on viability of macrophages.

Control		MS14 (100 mg/kg)	
Non-stimulator	LPS + fMLP	Non-stimulator	LPS + fMLP
0.20 ± 0.009	0.21 ± 0.01	0.20 ± 0.005	$0.19 \pm 0.003^*$

The effect of 5 days administration of MS14 on viability of macrophages (absorbance in MTT test). All data are represented as means \pm SEM.

*Denoted significant differences with control group $P < 0.01$.

MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

footpad after 24 h (Figure 4). Data were shown DTH reaction in control group was 24.78 and in MS14 group was 20.05 but this difference was not statistically significant ($P < 0.45$) and DTH reaction was not affected by MS14 at 100 mg/kg and 5 days administration.

Discussion

Macrophages probably damage normal tissues, in some activated inflammatory processes, because their anti-microbicidal products after release from these cells are not able to distinguish own tissues from microbes and many case undesirable inflammation.⁽¹⁰⁾ Inflammation is one important causes in various diseases which researchers and experts of various branches pay attention to its drug control as curative or symptom treatment.⁽¹⁹⁾

The key role of cytokines in pathogenesis of many diseases has been well known. It has been demonstrated that in some autoimmune diseases the Th1/Th2 cell response is shifted to a predominantly Th1 cell response, while an overwhelming Th2 response elicits allergic disorders.⁽²⁰⁾ Shokrgozar et al. in their research shown some pro-inflammatory cytokines (IL-2, IL-12 and $IFN\gamma$) have the main role in MS autoimmune disease and they present these cytokines as a risk factor for MS.⁽²¹⁾ Also Rosloniec et al. believed $IFN\gamma$ is a prime target for modulating autoimmunity, with the hypothesis being that if $IFN\gamma$ expression can be downregulated, then both of the Th pathways and the production of pathogenic autoantibody can be altered.⁽²²⁾ Since regulation of some cytokines such as IL-2 and $IFN\gamma$ could inhibit the onset and severity of some autoimmune disease, immunomodulators possess

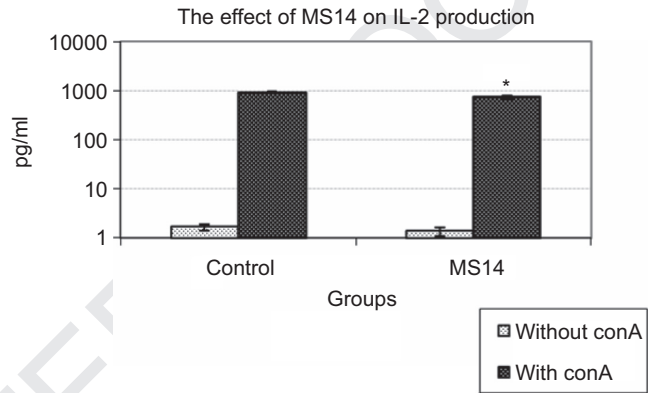


Figure 2. The effect of 5 days oral administration of MS14 (100 mg/kg) on concentration of interleukin-2 (IL-2) in mouse spleen lymphocytes culture. The lymphocytes were cultured at 2×10^5 and the IL-2 concentration was assayed by enzyme-linked immunosorbent assay kit. All data are represented as means \pm SEM. $P < 0.01$ denoted significant differences with control group at presence concanavalin A (ConA).

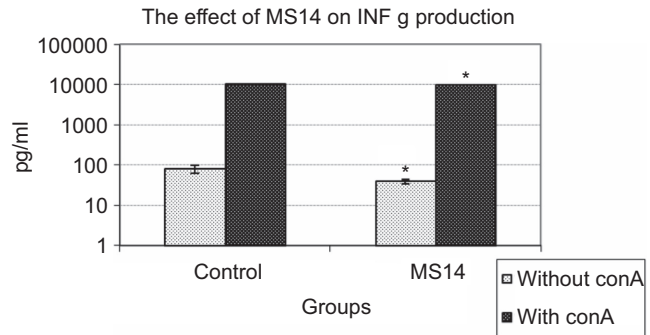


Figure 3. The effect of 5 days oral administration of MS14 (100 mg/kg) on concentration of $IFN\gamma$ of mouse spleen lymphocytes culture. The lymphocytes were cultured at 2×10^5 and the $IFN\gamma$ concentration was assayed by enzyme-linked immunosorbent assay kit. All data are represented as means \pm SEM. $P < 0.01$ denoted significant differences with control group.

an major important. Among a variety compounds with immunomodulator properties, herbal compounds are one of the proper goals as immunomodulators.

In this study, the anti-inflammatory and immunomodulatory effect of oral administration of MS14 on

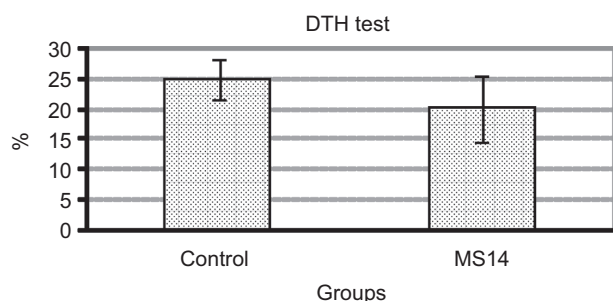


Figure 4. Delayed-type hypersensitivity (DTH) test. Mice were immunized subcutaneously (s.c.) with 3% sheep red blood cell (SRBC) suspension. After 5 days administration of MS14, the DTH reaction was elicited by s.c. injection of 3% SRBC suspension into one-hind footpad. Footpad swelling was measured 24h after footpad injection.

mouse peritoneal macrophages and spleen lymphocytes were investigated. According to oral administration (not peritoneal injection), the observed decrease could be attributed to some effects after drug absorption through gastrointestinal mucus rather probable direct toxic effect of MS14 on cells. More to the point, Hajihashemi et al. have shown that MS14 preparation up to a dose of 2500 mg/kg/day did not have any toxic effects when it was orally administrated.⁽¹³⁾ On the other hand, the results of MTT test in the present study shows that vital activity of macrophages has not been decreased in MS14 treated group, which negate cell death (as a probable cause of diminished responses) and confirm modulator mechanisms.

There are a few reports showing that various herbs can decrease and increase NO production,^(23,24) in special situations, the result of our study has shown that MS14 in 100 and 200 mg/kg for 6 days administration can decrease NO production of peritoneal macrophages.

The result of the other part of this study shows that the stimulators (PMA, fMLP, LPS) could powerfully increase the production of NO in both control and MS14 treated groups. This result indicates macrophages in both group are able to respond to stimulators normally, although the production value of NO in both status (with and without stimulators) was diminished in MS 14 treated group (about 77% when combination of stimulators were used). Considering that NO is produced by inducible synthetase NO (iNOS) enzyme in macrophages,⁽⁴⁾ MS14 may probably decrease iNOS gene expression or translation or it may somehow regulate enzyme activity. Also others suppositions are propounded which with their base design various trials and determine essential mechanism.

According to the above passage, this preparation possesses modulator mechanism that could be through an effect on NO production of peritoneal macrophages. We have also observed that MS14 can decrease IFN γ and same the result were obtained when splenic lymphocytes were stimulated with ConA.

A decrease in IL-2 production of the lymphocyte culture was observed only at the presence of ConA. Another part of the present study had shown that MS14 has not

affected DTH test in mice. IL-2 and IFN γ decrease by MS14 show that this preparation is useful for treatment of diseases in which these cytokine play pathologic roles, such as MS. On the other hand, no effect of MS14 on DTH test shows that this drug does not suppress all application of T-cells.

Our previous studies showed that MS14 create a decrease in some inflammatory cytokines as well (data unpublished) or it decreased the value of immunoglobulin M (IgM) in primary response without effect on IgG and secondary response.⁽²⁵⁾ Also in murine sepsis model, mice received MS14 were protected against *Candida albicans* and MS14 decreased mortality rate and colony count of kidney and blood at these mice.⁽²⁶⁾ At the other hand MS14 did not suppress the immune response totally e.g., when it had been given 100 mg/kg/day for 5 days to BALB/c mice, augments the IL-5 and IL-10 cytokines in supernatant of spleen lymphocytes culture.⁽²⁷⁾

Regarding the importance of inflammatory processes and role of Th1 cytokines pattern in autoimmune diseases, a non-toxic immunomodulator with the ability to decrease inflammatory and undesirable immune responses would be considered as a valuable tool. Nowadays, ample efforts are made in order to find immunomodulators with natural origin and with the ability of modulating immune responses with the least complication and less costs. Usually the general suppress of immune system activity has intense and dangerous complication, therefore an immunomodulator with less side effects and more specific action that especially decreases the desired action of immune responses, is preferred to be used and MS14 compound could be a suitable candidate for this manner.

In conclusion, it may be claimed that MS14 preparation could be considered as an immunomodulator with decreasing effect on some part of immune system activity. However, to obtain practical results in various diseases in which NO of macrophages and Th1 cytokines involves, more studies should be done.

Declaration of interest

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