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# Prevention of Experimental Autoimmune Vitiligo by Oral Administration of Mushroom Tyrosinase

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## ABSTRACT

**Experimental autoimmune vitiligo was induced by the intradermal injection of the purified mushroom tyrosinase emulsified in Complete Freund's Adjuvant (CFA) in female C57BL/6 mice. The onset of vitiligo was characterized by hair hypopigmentation and total melanocyte depletion in the basal layer of the epidermis. Oral administration of semipurified mushroom tyrosinase prevented experimental autoimmune vitiligo (EAV). Suppression of clinical and histological disease was observed when animals received mushroom tyrosinase. A decrease in lymphocyte proliferation, delayed type hypersensitivity responses, increase in humoral immunity of the mice may all suggest that the suppression of the disease is correlated with cellular immune responses suppression. Based on our data, it may be concluded that the oral administration of the mushroom tyrosinase may have practical implications in vitiligo.** *Iran. Biomed. J. 4: 31-36, 2000*

*Keywords*: Mushroom tyrosinase, Experimental autoimmune vitiligo, Oral tolerance.

## INTRODUCTION

Immunologic tolerance is defined as a state of specific immunologic unresponsiveness to an antigen after exposure to the antigen. The oral administration of autoantigens has been used to suppress the immune responses in several experimental animal models [1-5]. We have used oral tolerance as a means to suppress vitiligo. Vitiligo is an autoimmune condition that is found in 0.5-1% of the individuals worldwide. It is characterized by localized or diffused depigmented patches on the skin [6, 7]. The main target in this disease is melanocyte that could be affected by humoral [6-13] and cellular [14-17] immune responses. Several autoantigens on the surface of the melanocyte membrane have been reported which are the targets for anti-melanocyte antibodies in vitiligo [6, 13]. Tyrosinase has been identified as the principal autoantigen on the surface of melanocytes [6, 11, 18]. It is a 75 KDa copper containing enzyme, the key enzyme for the metabolism of melanin in pigmented cells and catecholamines in neuroendocrine systems [6]. Since, there is cross-reactivity between mushroom and mammalian tyrosinase [6]. We used mushroom tyrosinase as an antigen in our investigation. For the first time, we used the purified edible mushroom tyrosinase, emulsified in Complete Freund's Adjuvant (CFA), to induce the vitiligo in the genetically susceptible C57BL/6 mice [19], and we investigated the therapeutic benifits of the oral administration of the semipurified form of this enzyme. We found that immunization with mushroom tyrosinase induced experimental autoimmune vitiligo (EAV) and oral feeding of mushroom tyrosinase suppressed not only the onset of the disease but also the cell mediated immune responses to mushroom tyrosinase.

## MATERIALS AND METHODS

***Mice.*** Female C57BL/6 mice, 6 weeks of age, were purchased from Pasteur Institute, Karaj, Iran. They were housed under conventional conditions and were allowed free access to food and water.

***Isolation and purification of mushroom tyrosinase.*** Tyrosinase was prepared from edible fresh white mushroom (*Agaricus bispora*) as

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described by Nelson & Mason [20]. The purified tyrosinase was detected by PAGE [21] in the presence of standard mushroom tyrosinase (Sigma, USA). Enzyme activity was determined using the spectrophotometric method [22].

***Immunization of animals.*** Six-week-old female mice were injected intradermally (i.d.) on four sites of the back skin with 50 µg tyrosinase solution. The enzyme has been emulsified in 50 µl of CFA (Sigma, USA). Two weeks after the primary immunization, the mice were injected intra-peritoneally (i.p.) with 50 µg mushroom tyrosinase in 50 µl of Incomplete Freund's Adjuvant (IFA) (Sigma, USA) [23]. Control animals were also immunized with PBS+CFA.

***Oral administration protocol.*** Mice were orally administrated a dose of 200 µg of semipurified mushroom tyrosinase in phosphate buffered saline (PBS) solution, pH 7.2, for five times at intervals of 1 day immediately after the first immunization in a volume of 0.5 ml per mouse per feeding by means of a syringe filted with a ball-type feeding needle [24]. Control animals were fed only equal volumes of PBS.

***Histopathology.*** Hair hypopigmentation was observed four months after immunization. It was considered as a sign of disease. The animals were killed and the skin areas that contained hypopigmented hairs were taken and fixed in 10% formaldehyde in phosphate buffer saline, pH 7.2, and embedded in paraffin for light microscopic evaluation. Skin sections were stained with hematoxylin- eosin for further investigations.

***Measurement of serum antibody.*** Mice were bled from the retroorbital sinus, 7 days after i.p. boosting, and the sera were tested for anti-tyrosinase antibodies by using of Dot ImmunoBinding Assay (DIBA) [25, 26]. A nitrocellulose sheet (300 mm × 3,000 mm, pore size 0.2 µm, Schleicher & Schuell, Germany) was cut to an appropriate size by puncher. Nitrocellulose disks were put on the wells of flat-bottomed 96-well plates (Nunc, Denmark). Antigen solutions (1 mg/ml in TBS: 10 mM Tris Hcl containing 0.15 M Nacl, pH 7.6) were then dotted onto each disk (1ul) and allowed to dry at 37˚C for 30 min. The disks were then incubated in blocking buffer containing 1% bovine serum albumin (BSA, fraction V, Fluka, Switzerland) in TBS, at RT for 30 min. After washing with 0.02% Tween 20 in TBS (TBS-Tween) while shaking gently, the serial dilutions of sera were added to the wells and incubated at 37˚C for 2 h. After washing, anti-mouse Ig-Ap, Fab fragments produced in goat (Boehringer Mannheim Biochemica, Germany) diluted 1/1,000 in TBS, was added to the wells and incubated at 37˚C for 30 min. After washing, the color developing solution (BCIP-NBT, Sigma) was added and kept till spots appeared (5-10 min). The wells were washed with $dH_2O$ to stop the reaction.

***DTH responses.*** Cell mediated immunity was assessed 7 days after i.p. boosting by measuring the specific increment in footpad thickness 24 h after intradermal challenge with 50 µg mushroom tyrosinase in 0.05 ml of saline (0.15 M) [27].

***In vitro proliferation assay.*** Fourteen days after the second immunization, five mice from each experimental group were killed. The erythrocyte depleted spleen cell suspensions were prepared in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) containing 100 ug/ml penicillin, 100 µg/ml Streptomycin, 10% FCS (Sigma, USA) and 200 mM L- glutamine (Sigma, USA). The cells were cultured with 20 ug mushroom tyrosinase and Con A (Sigma, USA) at the cell concentration of $0.5 \times 10^6$ cells/well in round-bottomed 96-well plates (Nunc, Denmark) for 3 days and pulsed by adding 1 µCi $^3$H-thymidine for the final 18 h. Cells were then harvested and filters counted in the presence of 2 ml of Scintillation fluid (Sigma, USA). The results were expressed as stimulation indices, which is defined as the counts in the antigen containing wells devided by counts in the medium-containing wells.

***Determination of $CD4^+/CD8^+$ lymphocytes ratio.*** To examine further, the type of T cell subset which is involved in antigen recognition, the spleen cells were harvested and cultured as above except the cells cultured in 24-well plates containing 1 ml ($2 \times 10^6$ cells), at the end of stimulating period, the cells were harvested and used for immunophenotyping as it follows: $1 \times 10^6$ cells of each well were stained with 4 ul of either CD3-FITC/CD4-PE or CD3-FITC/CD8-PE monoclonal antibodies (Sigma, USA). They were incubated at 4˚C for 30 min. The samples were washed with 3 ml PBS, pH 7.2 and results read with Flowcytometry system (Facscalibore, Becton Dickinson, San Jose/CA).



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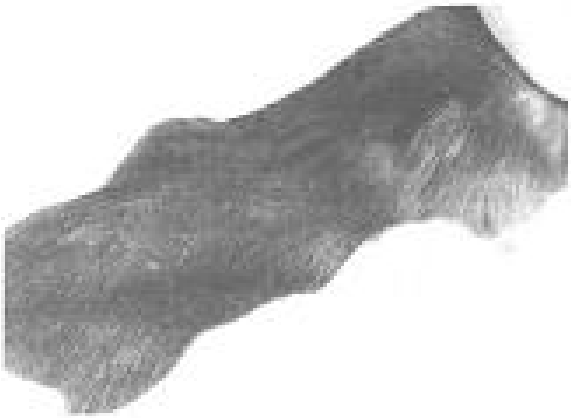

**Fig. 1.** Experimental autoimmune vitiligo after two immunizations with mushroom tyrosinase.

***Statistical analysis.*** The statistical analysis was performed by students *t-test*, $P<0.05$ being considered significant.

## RESULTS

***Histopathological findings.*** Our data showed that four months after the second immunization of the female C57BL/6 mice with mushroom tyrosinase emulsified in CFA, they developed hypopigmented patches on the back hairs (Fig. 1).

The histopathological study of the hematoxylin-eosin stained sections revealed a fragment of the skin which showed acanthosis with total melanocyte depletion in the basal layer of the epidermis and a foreign body granoluma in the dermis composed of epithelioid histiocytes, multinucleated giant cells and mononuclears with dense fibro-collagenous bundles. The control group of mice that were immunized with PBS+CFA have not shown the histological changes described above (Fig. 2). Our results indicated that the clinical expression of the disease was suppressed in animals fed with tyrosinase solution.

***Effect of feeding mushroom tyrosinase on the levels of anti-tyrosinase specific antibody.*** Oral administration of mushroom tyrosinase caused a significant increase ($P<0.05$) in the levels of anti-tyrosinase specific antibodies (Fig. 3). In our investigation, we also indicated that oral administration of mushroom tyrosinase without immunization can not by itself induce systemic anti-mushroom tyrosinase antibodies (data not shown).

**Fig. 2.** Skin histopathology of C57BL/6 mice. (a) Inflammatory responses as a result of immunization with mushroom tyrosinase emulsified in CFA have been observed in the dermis. The architecture of the epidermis is disorganized with the loss of the melanocytes (Hematoxylin and eosin, original magnification ×40). (b) Skin of control animal composed of the thin epidermis and the normal dermis, in the basal layer 10- 12% scattered melanocytes are visible.

***DTH responses after oral administration of mushroom tyrosinase***. DTH responses were performed *in vivo* to determine the effect of feeding mushroom tyrosinase on T cell responses. As it is shown in (Fig. 4), oral administration of mushroom tyrosinase caused a significant reduction ($P<0.05$) in DTH responses in comparison to the corresponding control mice.

***In vitro proliferative responses.*** The effect of oral administration of mushroom tyrosinase on the *in vitro* T cell responses was also examined. The results (Fig. 5) demonstrated that feeding of mushroom tyrosinase caused a pronounced decrease of the proliferative response to mushroom



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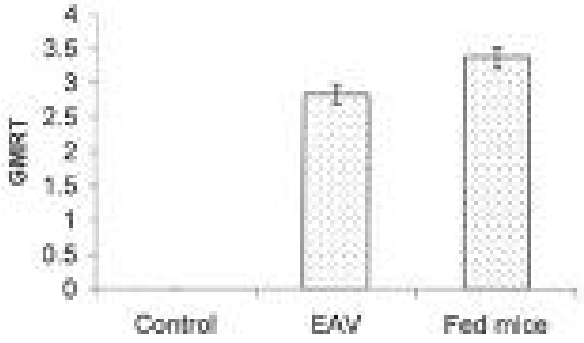


**Fig. 3.** Effect of feeding of mushroom tyrosinase on the production of serum antibody. Mice were fed immediately after the first immunization (fed mice). Experimental Autoimmune Vitiligo, firstly, immunized with purified mushroom tyrosinase accompanied by CFA and immediately fed with PBS. Seven days after the second immunization, mice were bled and the sera were tested for anti-tyrosinase specific antibody using Dot ImmunoBinding Assay. The data are presented as the Geometrical Mean of Reversed Titer (GMRT), n =15 per group. Control animals were fed with buffer only.

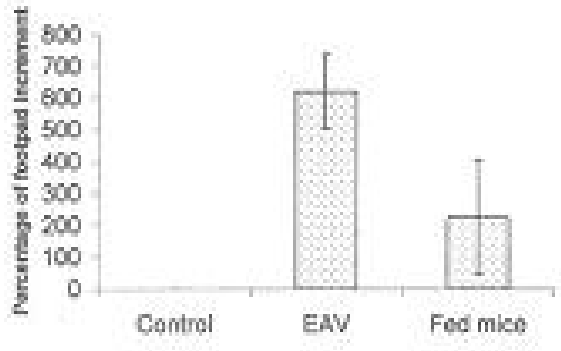


**Fig. 4.** Effect of oral administration of mushroom tyrosinase on DTH responses. Female C57BL/6 mice were fed immediately after the first immunization (fed mice) and DTH responses were measured. Data are presented as the mean of the specific increment in footpad thickness, 24 h after intradermal challenge with mushroom tyrosinase, n =15 per group.

tyrosinase ($P<0.05$). No statistically significant differences were found on the proliferative responses to Con A.

***Effect of oral feeding of mushroom tyrosinase on CD4$^+$/CD8$^+$ lymphocytes ratio.*** Our data demonstrated (Fig. 6) that oral administration of mushroom tyrosinase caused increasing in CD4$^+$ /CD8$^+$ lymphocytes ratio ($p<0.05$).

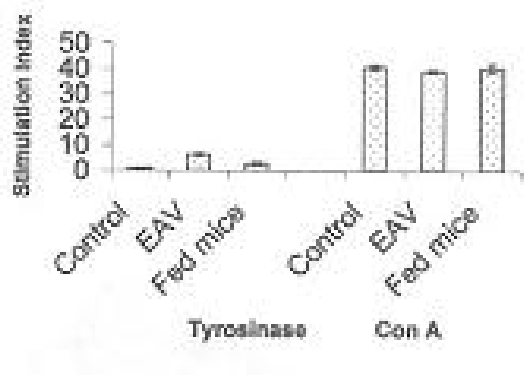


**Fig. 5.** Effect of oral administration of mushroom tyrosinase on *in vitro* splenocyte responsiveness to mushroom tyrosinase. Columns represent the mean of stimulation indices of $^3$H-thymidine uptakes in each group to *in vitro* challenge with mushroom tyrosinase. Results represent five mice per group.

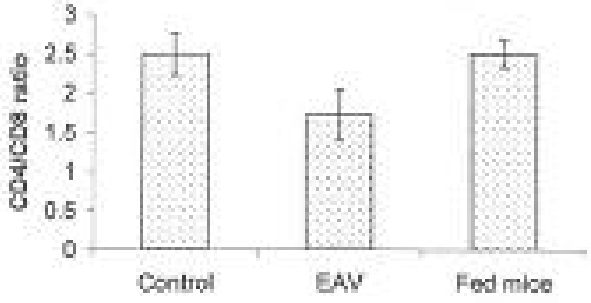


**Fig. 6.** Effect of oral feeding mushroom tyrosinase on CD4$^+$/CD8$^+$ lymphocytes ratio in splenocyte culture of mice fed immediately after the first immunization.

## DISCUSSION

The oral administration of antigen has been widely studied as a means of suppressing the autoimmune diseases including experimental autoimmune uveoretinitis in Lewis rats fed with S-antigen [1], experimental autoimmune encephalomyelitis in Lewis rats fed with myelin basic protein [3], and arthritis in Lewis rats fed with type II collagen [5]. We have demonstrated, for the first time, that immunization with mushroom tyrosinase emulsified in CFA induces EAV in female C57BL/6 mice. The induction of the vitiligo by immunization with mushroom tyrosinase suggests that the tyrosinase could be an effective autoantigen responsible for the genesis of the vitiligo. Destruction of melanocytes in the basal layer of the epidermis and the presence of



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inflammatory cells in the dermis of EAV indicates that the destruction of melanocytes is probably caused by the mononuclear cells. In addition, the absence of overt infiltrates in the epidermis may be a consequence of dispersion of the inflammatory cells throughout the basal layer of the epidermis. Our data showed that immunization with purified mushroom tyrosinase accompanied by adjuvant caused a decrease in $CD4^+/CD8^+$ lymphocytes ratio. This finding demonstrates that $CD8^+$ lymphocytes may have been involved in the pathogenesis of the disease.

In this investigation, we have also shown, for the first time, that oral feeding of mushroom tyrosinase suppresses clinical and histological symptoms of the disease and profoundly suppresses T cell proliferative response to mushroom tyrosinase, and it increases anti-mushroom tyrosinase antibody production, which is consistent with orally induced suppression of immune responses for other antigens [26]. The lymphocyte proliferation and the DTH measurements demonstrated that suppression of EAV by feeding of mushroom tyrosinase was closely linked to the suppression of cellular immune responses to mushroom tyrosinase. Our other results are an increasing in antibody responses and $CD4^+/CD8^+$ lymphocytes ratio that remained to be elicited. Many groups have reported that oral feeding of antigen induces a Th2-dominant response in systemic organs such as the spleen and mucosal sites [28]. Since, organ-specific autoimmune diseases are mediated by Th1 subtypes [28], it may be concluded that immune deviation might be considered as a therapeutic approach capable of modulating the immune responses of autoantigen-specific T cells involved in pathogenic immune reactions.

Our further investigation is aimed to determine the potential mechanisms involved in the induction of oral tolerance in vitiligo and the pattern of cytokine reflecting the Th1/Th2 balance.

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