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Intravenous Injection of Myelin Oligodendrocyte Glycoprotein-coated PLGA Microparticles Have Tolerogenic Effects in Experimental Autoimmune Encephalomyelitis

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ABSTRACT

The abnormal function of the T lymphocytes causes a range of autoimmune diseases, particularly multiple sclerosis; hence, several methods have been used to treat these disorders through the induction of antigen-specific tolerance in T cells. The present study aims to use a simple and low-cost method to produce poly (lactic-co-glycolic acid) (PLGA) nanoparticles for carrying antigens and inducing antigen-specific tolerance.

In this study, PLGA nanoparticles were produced using the water/oil/water (W/O/W) method. The myelin oligodendrocyte glycoprotein (MOG) peptide and ovalbumin peptide(OVA) were covalently bound to the synthetic PLGA nanoparticles in the presence of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) and were injected to six groups of C57BL/6 mice one week before the induction of the experimental autoimmune encephalomyelitis (EAE) intravenously or subcutaneously; one group was considered as control; finally, immunologic responses including delayed-type hypersensitivity (DTH) response and lymphocyte proliferation were investigated.

The results showed that the intravenous injection of microparticles containing MOG peptides before the development of the EAE model, not only could delay the incidence of syndrome, but also increase the antigen-specific tolerance. Moreover, a reduced delayed-type hypersensitivity response was observed in the mice primed with microparticles containing MOG peptides. In addition, a reduced spleen lymphocyte proliferation was found in the same mice when challenged with antigens.

The present study proposes a simple, inexpensive, effective and safe method for preparing MOG-conjugated PLGA microparticles with immune tolerance properties that can be used in the treatment or reducing clinical syndromes of EAE model.

Keyword: Experimental autoimmune encephalomyelitis; Immune tolerance; Microparticles; poly (Lactic-co-glycolic acid)

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INTRODUCTION

Autoimmune diseases have been known as a major

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cause of death in the world.[1] In fact, a lack of regulatory T (T_{Reg}) cells results in severe autoimmunity in both mice and humans[2]. Hence, in general, current standard methods for the treatment of autoimmune diseases are focused on immune suppression; however, the nonspecific nature of this type of treatment usually put the patient at risk of opportunistic infections.[2,3] According to this, nowadays, antigen-specific tolerance induction is considered as one of the preferred methods for the treatment of T cell mediated autoimmune diseases,[4] however there has been little success.

In this context, methods used to induce peripheral T cell tolerance such as, soluble peptides inoculation, modified peptide ligands, using anti-CD3 antibody and co- stimulatory molecules blocking have been unsuccessful.[2,5,6] On the other hand, the use of hematopoietic stem cells or immune therapy with regulatory T cells due to the inability to produce a sufficient number of stem cells and providing stable and specific Treg cells did not succeed.[4] Another promising option for inducing long-term T cell tolerance is intravenous injection of peptides attached to the splenic leukocytes using 1-ethyl-3-(3 dimethylaminopropryl) carbodiimide hydrochloride (EDCI),[7-9] althoughthe mechanism of tolerance induction by spleen cells attached to the antigen has not been well documented. In spite of this, the results of this method have led to developing a new class of vaccines, which promote immunological tolerance referred as tolerogenic vaccine.[10-12]

Because of Poly(lactic-co-glycolic acid) (PLGA) biocompatibility and FDA approval for its use,[13,14] we tried to present a simple and low-cost method to produce myelin oligodendrocyte glycoprotein (MOG) coated PLGA nanoparticles as a tolerogenic vaccine for experimental autoimmune encephalomyelitis (EAE) treatment.

MATERIALS AND METHODS

Preparation of Microparticles

PLGA (50:50, 50.000-75.000, 430471 - Sigma-Aldrich, UK) microspheres were prepared aseptically by a double emulsion solvent evaporation method (W/O/W) at room temperature. Briefly, based on previous investigations,[15] 1 mL of phosphate buffer saline (pH 7.4) containing N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB, MERCK,USA) (0.2%, w/v) was suspended in 10 mL of 4% w/v PLGA solution in ethyl acetate and sonicated for 1 min at 50 watt (W) in an ice-bath. This water- in -Oil (W/O) emulsion was added into 20 mL of 2% w/v aqueous polyvinyl alcohol (88% hydrolyzed, 20.000-30.000, Achros) and mixed at high speed (4000 rpm) using mechanical stirrer. The organic solvent was allowed to evaporate and the resulted microspheres were washed and collected by a mild centrifugation (4000 rpm, 5 min). All the supernatant were collected and washed twice with phosphate buffer saline (PBS) and passed through filter paper (amicon 8010,MERCK,USA) with different cut off (100 and 30 kilo dalton).Three fractions were collected and lyophilized. The nanoparticles that passed through 30 KD paper designated $PLGA_{100}$, the nanoparticles that could not pass through 30 KD paper designated $PLGA_{300}$ and the nanoparticles that could not pass through 100 KD paper designated $PLGA_{500}$.

Peptides and Reagents

Synthetic peptides including myelin oligodendrocyte glycoprotein (MOG_{35-55}), (MEVGWYRSPFSRVVHLYRNGK), ovalbumin ($OVA_{323-339}$) (ISQAVHAAHAEINEAGR), polymers such as poly (dl-lactide-co-glycolide; 50:50), M.W 50,000–75,000 KD (used for preparation of the microspheres), Pertussis toxin, were purchased from SIGMA, UK). All other chemicals and reagents were analytical grades and purchased from local suppliers.

Conjugation of Antigen to PLGA Microparticles

Based on established methods[16], microparticles at a final of 1% solids were pre-activated in a mixture containing EDCI (4 mg/mL final), N-hydrosulfosuccinimide (Sulfo-NHS) (50 mM final). The pre-activated nanoparticles were incubated for 2 h on a rotary wheel at room temperature (RT). Antigens such as $OVA_{323-339}$ or myelin MOG_{35-55} peptides were added to the pre-activated nanoparticle mixture (1 mg/mL) and further incubated for 72 h to achieve the desired antigen coupling rates. Following antigen incubation, unbound sites on nanoparticles were saturated by adding excess glycine (7 mg/mL final) and further incubated for 30 min. The conjugation mix was then dialyzed in 10–14 KDa molecular weight cut-off (MWCO) membrane against phosphate buffered solution (PBS, pH = 7.2) at 4°C overnight for unbound peptide separation. The peptide coupled microspheres could not be sterilized by filtration. Terminal

sterilization of the microspheres (UV-irradiation) may lead to degradation of the peptide. Thus, microspheres were prepared aseptically and stored in 4°C after lyophilization.

Determination of Conjugation Efficiency

The conjugation efficiency of peptide antigens were determined by quantifying the amount of unconjugated protein in the supernatant from the nanoparticles formulations produced by the above procedures.[15] A sample aliquot of the nanoparticle conjugate was ultra-centrifuged (Beckman TLA-100.3, Beckman Coulter, Fullerton, USA) at 70,000 rpm for 20–30 min at 4 °C. The supernatant was then collected and analyzed using the Micro BCA protein assay kit according to the manufacturers' instructions (Pierce Micro BCA protein assay, Thermo Fisher Scientific, USA).

Mice

The groups of inbred, female C57BL/6 mice, 6-8 weeks old, were purchased from Pasteur Institute of Iran. Each group consisted of five mice, which were housed in a standard poly-propylene cage (anti-acid, anti-base cages). The animals were kept under the standard conditions (a cycle of 12/12 h light/dark and a temperature of 20-22◦C) with free access to water and autoclaved standard mouse chow. Animal care and treatment were conducted in conformity with the guideline of Animal Care and Research Committee of Tarbiat Modares University.

Mice Treatment with Antigen-coupled PLGA

Animals received intravenous or subcutaneous injections of approximately 2 mg nanoparticles containing about 20 µg of peptide in sterile PBS on day -7 (Figure 1). Three groups received $PLGA_{500}$ - MOG_{35-55} , $PLGA_{500}$- $OVA_{323-339}$ microparticles and $PLGA_{100}$- MOG_{35-55} nanoparticles intravenously and three groups $PLGA_{500}$ - MOG_{35-55} , $PLGA_{500}$- $OVA_{323-339}$ microparticles and $PLGA_{100}$- MOG_{35-55} nanoparticles subcutaneously. One group of mice had no injection and was used as naive mice (Table 1).

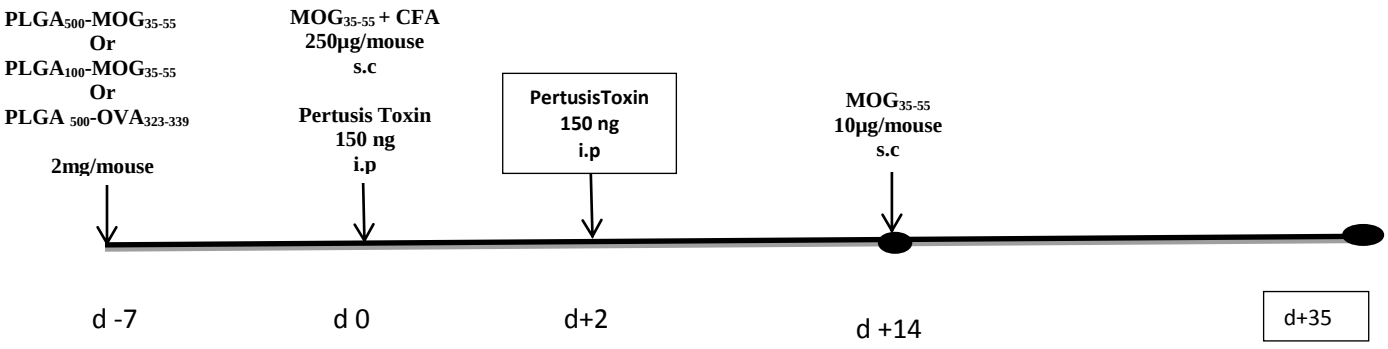
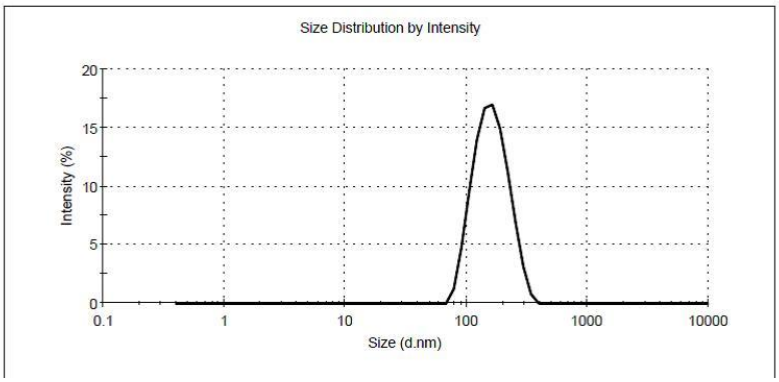


Figure 1. Immunization and challenging time:
Animals received intravenous or subcutaneous injections of approximately 2 mg nanoparticles comprising about 20µg of peptide in sterile phosphate buffered solution on day -7. Peptide-induced experimental autoimmune encephalomyelitis (EAE) was induced by immunization with myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) in day 0. On day +14 delayed type hypersensitivity (DTH) responses were examined

(a)

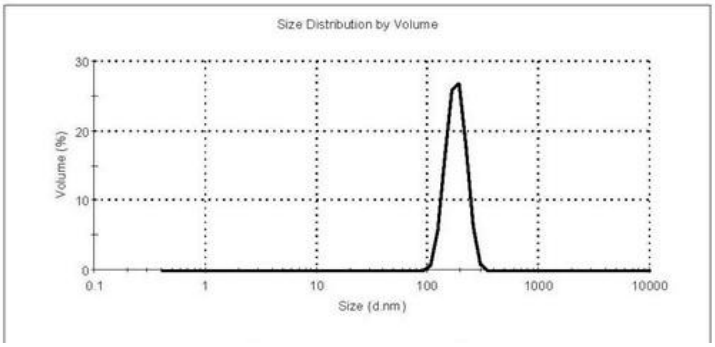
		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 151	Peak 1:	166	100.0	52.9
PdI: 0.100	Peak 2:	0.00	0.0	0.00
Intercept: 0.899	Peak 3:	0.00	0.0	0.00



(b)

Results

		Diam. (nm)	% Volume	Width (nm)
Z-Average (d.nm): 389	Peak 1:	181	100.0	36.3
PdI: 0.602	Peak 2:	0.00	0.0	0.00
Intercept: 0.578	Peak 3:	0.00	0.0	0.00



(c)

Results

		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 521	Peak 1:	228	100.0	36.4
PdI: 0.538	Peak 2:	0.00	0.0	0.00
Intercept: 0.577	Peak 3:	0.00	0.0	0.00

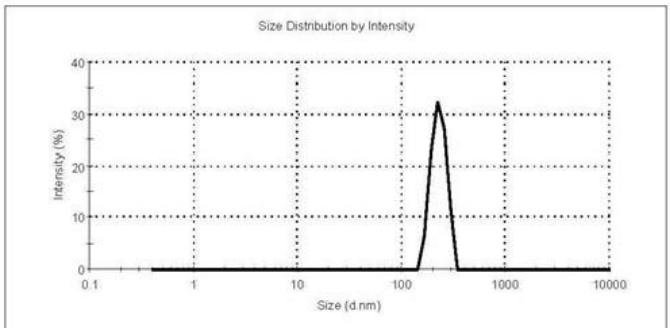


Figure 2.Characterization of different particle preparations by dynamic light scattering (DLS) technique used in Tolerance Induction .(a) 100 nm nanoparticles, (b) 300 nm nanoparticles and (c) 500 nm nanoparticles

Table 1. Mice Treatment schedule with different antigen-coupled poly(lactic-co-glycolic acid) (PLGA) particles used in **Tolerance Induction: Six groups of mice received intravenous or subcutaneous injections of approximately 2 mg nanoparticles containing about 20 µg of peptide in sterile phosphate buffered saline (PBS) on day -7. One group had no treatment and was used as control group in delayed- type hypersensitivity (DTH) response and spleen lymphocyte proliferation test. $PLGA_{500}$-$OVA_{323-339}$ nanoparticles were used as peptide control in this study.**

Group	Group name	Nanoparticles used for priming (in day -7)	Route of nanoparticles administration	EAE induction (in day 0)	DTH response test (in day+14)	Splenocyte proliferation test (in day+14)
1	Test	$PLGA_{500}$-MOG_{35-55}	Intravenous	Yes	Yes	Yes
2	Test	$PLGA_{100}$-MOG_{35-55}	Intravenous	Yes	Yes	Yes
3	Control	$PLGA_{500}$-$OVA_{323-339}$	Intravenous	Yes	Yes	Yes
4	Test	$PLGA_{500}$-MOG_{35-55}	Subcutaneous	Yes	No	No
5	Test	$PLGA_{100}$-MOG_{35-55}	Subcutaneous	Yes	No	No
6	Control	$PLGA_{500}$-$OVA_{323-339}$	Subcutaneous	Yes	No	No
7	Untreated	-	-	No	Yes	Yes

EAE: experimental autoimmune encephalomyelitis

Induction of Peptide-induced EAE

Peptide induced EAE was induced in C57/BL6 mice as previously described.[17,18] Briefly, mice were primed with an emulsion containing 1 mg/mL MOG_{35-55} and Freund Complete Adjuvant (CFA) containing 4 mg/mL mycobacterium tuberculosis H37Ra (Difco, The Netherlands). 100 µL of emulsion was injected subcutaneously (S.C.) in three sites on the flank of each mouse. The mice were observed daily and clinical scores were assessed in a blinded fashion. The 0-5 scales definitions were as follows: 0, no abnormality; 1, limp tail or hind limb weakness; 2, limp tail and hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; and 5, moribund. The mean daily clinical scores were reported. To prevent the death of paralyzed mice because of the lack of food and water those mice were given easier access to food and water. On day + 14 some mice were used for DTH response and spleen lymphocyte proliferation assay (Table 1).

Delayed- Type Hypersensitivity

To determine if antigen-specific T cell tolerance was induced by peptide-PLGA administered on day -7, DTH responses were examined on day +14.[19] After MOG_{35-55} challenge, ear thickness was determined. Immediately thereafter, DTH responses were elicited by injecting 10 µg of peptide in 10 µL of PBS into the dorsal surface of the ear using a 100 µL Hamilton syringe fitted with a 30 gauge needle. The increase in ear thickness compared to pre-challenge measurements was determined by a Mitutoyo model 7326 engineer's micrometer (General ULTRA TECH, Japan). Results were reported in millimeter (mm).

Spleen Lymphocyte in vitro Proliferation Assay

For cell activation and proliferation assays, at the indicated times following disease induction, the mice were sacrificed by cervical dislocation and splenocytes were harvested, counted, and cultured in 96-well microtiter plates at a density of 5×10^5 cells/well in a total volume of 100 µL of RPMI 1640 medium (Gibco,ThermoFisher, USA) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine); finally 200 µL RPMI1640 medium containing 10 µg/mL MOG_{35-55} was added to all wells. Cells were cultured at 37°C for 72 h. Cell proliferation was measured by BrdU Cell Proliferation Assay Kit (Sigma-Aldrich, UK).

Equipment

The size distribution and surface charge of PLGA nanoparticles were measured on Zetasizer Nano (Malvern, MRK825-02, UK), for dynamic light scattering (DLS) and scanning electron micrographs (SEM) a KYKY electron microscope (model EM-3200 ,China) was used at 26 kV.

Statistical Analyses

In this study each experiment was performed in duplicate or triplicate and one-way analysis of variance (ANOVA) was used to determine the statistical significance (p<0.05) between values of the test and

control groups. The data were analyzed using Prism software (Prism PT. Koneksi Integrasi, Indonesia) and the results are expressed as the mean ± standard error (mean± SE).

RESULTS

Nanoparticle Characterization

According to SEM images, the nanoparticles produced by the W/O/W method were spherical in shape and had a smooth surface. $PLGA_{500}$ particles contained microparticles of various sizes, ranging from 400 to 1500 nm. $PLGA_{100}$ particles contained nanoparticles smaller than 100 nm. The polydisperse index (PDI) of $PLGA_{100}$, $PLGA_{300}$ and $PLGA_{500}$ particles was 0.1, 0.53, and 0.85, in respective order. The zeta potential for $PLGA_{100}$, $PLGA_{300}$ and $PLGA_{500}$ particles was -14.1, -5.65 and -18.0. According to the DLS results, the mean size of $PLGA_{100}$, $PLGA_{300}$ and $PLGA_{500}$ particles was 151 nm, 389 nm and 521 nm (Figure 2, Table 2).

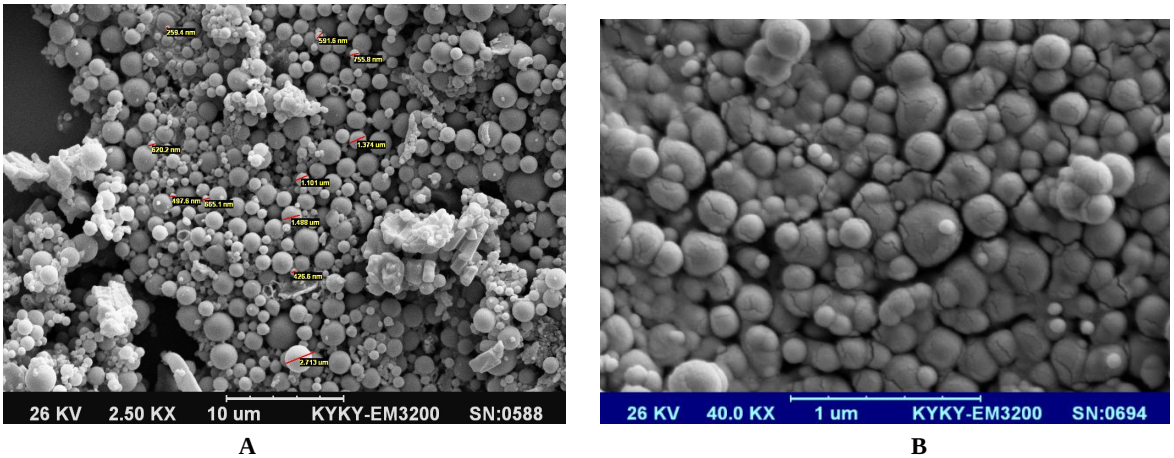
After the binding of the peptides to the PLGA nanoparticles, the nanoparticles' surface properties did not change and their surface remained smooth and no nanoparticle aggregation was observed (Figure 3). The efficiency rate of MOG peptides binding to $PLGA_{500}$ microparticles and $PLGA_{100}$ nanoparticles was %9.56 and %25.85, respectively. The efficiency rate of the binding of the OVA peptides to $PLGA_{500}$ and $PLGA_{100}$ was% 8.59 and %24.2 (Table 3).

Table 2. Characterization of the PLGA particles that were separated by filter papers tolerance induction. Three different fraction of nanoparticles were characterized based on particle size, zeta potential and polydispersity index (PDI)

Particle	average size (nm)	ζ – potential (mV)	Pdi
$PLGA_{100}$	151.2	-14.1	0.100
$PLGA_{300}$	371.8	-18.0	0.854
$PLGA_{500}$	521.1	-5.65	0.538

Table 3. Comparison of coupling efficiency in different poly(lactic-co-glycolic acid) (PLGA) particles used in Tolerance Induction . Ovalbumin (OVA) and myelin oligodendrocyte glycoprotein(MOG) covalently linked to PLGA100 and PLGA500 nanoparticles with different efficiency

Particle	average size (nm)	ζ – potential (mV)	MOG_{35-55} Coupling Efficiency (%)	MOG_{35-55} (µg peptide/mg particle)	$OVA_{323-339}$ Coupling Efficiency (%)	$OVA_{323-339}$ (µg peptide/mg particle)
$PLGA_{500}$	521.7	-5.65	9.65±0.21	9.60	8.59±0.23	8.60
$PLGA_{100}$	151.2	-14.1	25.85±0.2	25.80	24.20±0.01	24.0



A B

Figure 3. Comparison of surface morphology of 500 nanometer PLGA particles before and after peptide conjugation. The SEM imaging of $PLGA_{500}$ microparticles(A)and $PLGA_{500}$-MOG_{35-55} microparticles(B) revealed no aggregation after covalent peptide binding.

DTH Response

The results of challenging C57BL/6 mice with MOG antigen in the ear on day +14 showed an significant ear thickness increase of 0.4 mm in the mice primed with either $PLGA_{100}$-MOG_{35-55} or $PLGA_{500}$-$OVA_{323-339}$, and a maximum thickness increase of 0.2 mm at the challenge site in the mice primed with $PLGA_{500}$-$MOG_{35-55;}$ this result was statically significant ($p \leq 0.05$) (Figure 4a).

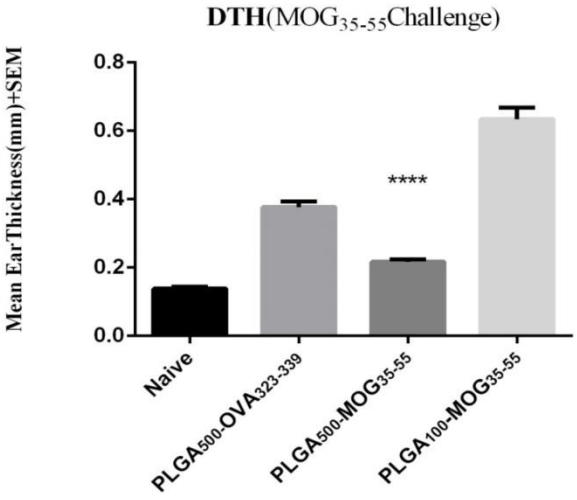
Spleen Lymphocyte Proliferation Assay

The lymphocytes of the mice primed with $PLGA_{500}$-$OVA_{323-339}$ and $PLGA_{100}$-MOG_{35-55} showed a higher proliferation, when immunized with MOG antigen compared to the mice primed with $PLGA_{500}$-MOG_{35-55} and the naive mice, as the optical absorption measured by the Brdu method was about 0.5 for the naive(untreated) mice and 0.64 for the mice primed with $PLGA_{500}$-MOG_{35-55}, and 1.9 and 1.8 for the mice primed with $PLGA_{500}$-$OVA_{323-339}$ and $PLGA_{100}$-MOG_{35-55} ; this result was statically significant ($p \leq 0.05$) (Figure 4b).

Peptide-induced EAE (Clinical Evaluation)

In the mice primed intravenously with $PLGA_{500}$-$OVA_{323-339,}$ the symptoms of the disease began from day +10 and reached their maximum on days +14 and +15. In the animals primed intravenously with $PLGA_{100}$-$MOG_{323-339,}$ the syndromes began from day +10 and reached their maximum on day +14. In the animals primed intravenously with $PLGA_{500}$-MOG_{35-55}, the syndromes began from day +14 and reached their maximum on day +18. The maximum mean clinical score (M.C.S) was about 3.5 for the mice primed with $PLGA_{500}$-$OVA_{323-339}$ and $PLGA_{100}$-MOG_{35-55} and gradually decreased and was stable at around 2.5. The maximum M.C.S was about 2.5 for the mice primed with $PLGA_{500}$-MOG_{35-55} , then gradually decreased and was fixed at around 1.8; as shown in Figure 5, this difference was statistically significant ($p \leq 0.05$).

The results were different for the mice primed subcutaneously, as in the mice primed with $PLGA_{500}$-MOG_{35-55} and $PLGA_{100}$-MOG_{35-55}; the clinical syndromes began on day +7, which was 3 days earlier than in the mice primed subcutaneously with $PLGA_{500}$-$OVA_{323-339}$.



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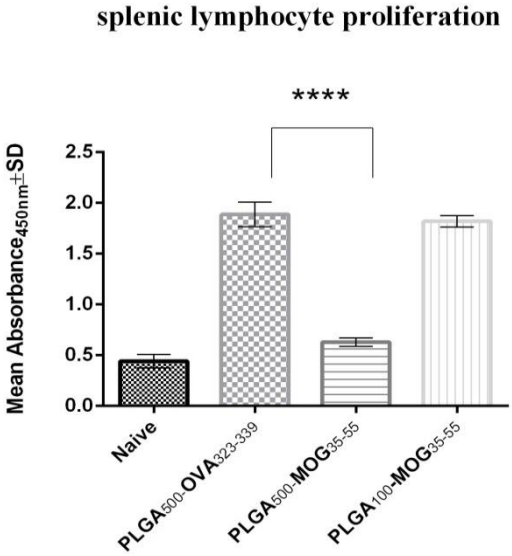

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Figure 4. Immune response to tolerance induction by peptide-coupled poly(lactic-co-glycolic acid) (PLGA) particles.
At day 14, 24 h delayed- type hypersensitivity(DTH) responses to ear challenge with 10 µg of MOG_{35-55} were determined in 4-5 selected mice from the PLG_{500}-MOG_{35-55} , $PLGA_{100}$-$MOG_{35-55,}$ and $PLGA_{500}$-$OVA_{323-339}$ treated groups(A), Spleen Lymphocyte proliferation assay(B), proliferative responses of spleen T cells were determined following 72 h in vitro stimulation with peptide by Brdu kit (B). Responses in PLGA500-MOG35-55-treated mice were significantly less than those in $PLGA_{500}$-$OVA_{323-339}$ and $PLGA_{100}$-MOG_{35-55} treated controls ($p \leq 0.0001$, ANOVA).

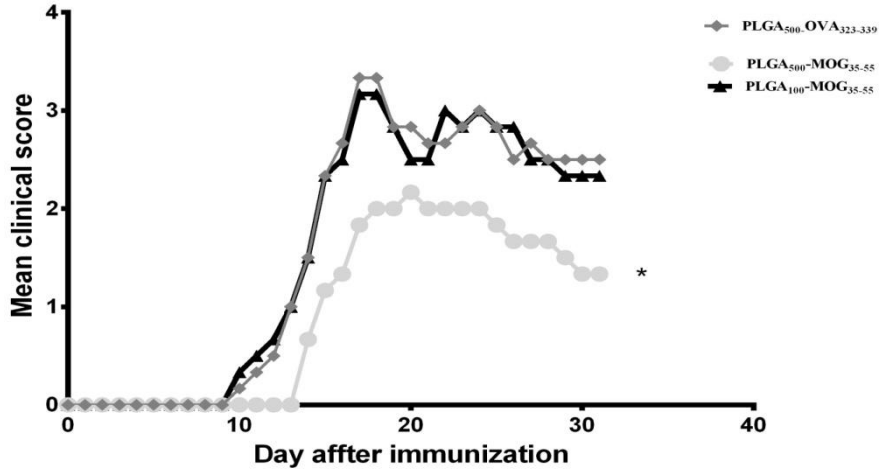

Figure 5. The effect of different peptide-coupled particles on mean clinical score (MCS) in experimental autoimmune encephalomyelitis mice

Groups of 6-8 week-old C57B/L6 mice were injected intravenously with 2 mg of various nanoparticles coupled to $OVA_{323-339}$ or MOG_{35-55} at 7day prior to induction of EAE by subcutaneous immunization with MOG_{35-55}/ CFA. Disease symptoms were scored by daily assessment of mean clinical score for the next 35 days. All experimental groups consisted of 4-6 mice and representative of three separate experiments .Mean clinical scores for the mice primed intravenously with MOG_{35-55} coupled to $PLGA_{500}$ were significantly lower than scores for mice that primed with other nanoparticles. ($p \leq 0.05$, ANOVA)

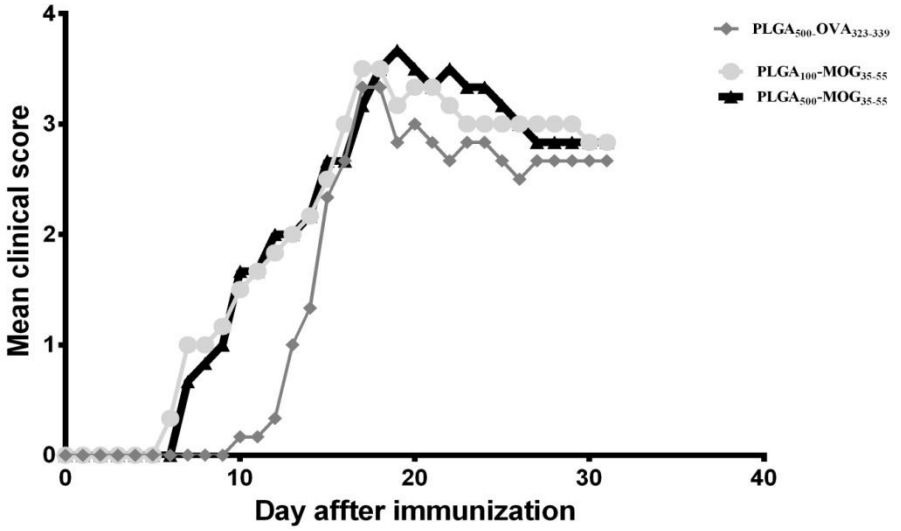

Figure 6. The effect of the injection site in the inhibitory effects of nanoparticles: Groups of 6-8-week old C57B/L6 mice were injected subcutaneous with 2 mg of various nanoparticles coupled to ovalbumin or myelin oligodendrocyte glycoprotein peptide (OVA323-339 or MOG 35-55) at 7day prior to induction of experimental autoimmune encephalomyelitis (EAE) by subcutaneous immunization with MOG35-55 and Freund's Complete Adjuvant (CFA) .Disease symptoms were scored daily by assessment of mean clinical score for the next 35 days. All experimental groups consisted of 6 mice and representative of three separate experiments. Mean clinical scores for the mice primed subcutaneously with MOG 35-55 coupled to either PLGA500 or PLGA100 were significantly higher than scores for mice that did not primed (p>0.17,ANOVA).

However, the maximum M.C.S was about 3.6 in the mice primed with $PLGA_{500}$-MOG_{35-55} and $PLGA_{100}$-MOG_{35-55} , which is slightly higher than in the mice primed with $PLGA_{500}$-$OVA_{323-339}$; as presented in Figure 6, this differences is not statically significant (*p*-value=0.17).

DISCUSSION

The present study used a simple and low-cost method to produce MOG_{35-55} peptide-coated PLGA microparticles, which not only can induce tolerance in EAE model, but also can be used as a tolerogenic vaccine for reducing clinical syndromes of this disease. In this regard, most studies have used nanoparticles for inducing an active and specific immune response against a pathogen in order to develop vaccines;[10,20,21] however, the present investigation, used PLGA nanoparticles to induce immune tolerance in the EAE model. A study conducted by Getts et al[22] showed that 500 nm carboxylated polystyrene microparticles that proteolipid protein ($PLP_{139-151}$) peptides are covalently bound to them could induce tolerance in the EAE model; but given that polystyrene particles have no clinical application, FDA-approved biocompatible PLGA nanoparticles may be a good alternative for carrying antigens.[13,23,24]

In current study, nanoparticles were separated by centrifugation and filtration; Three groups of separated nanoparticles were formed, including a group of nanoparticles with a diameter of less than 100 nm, a second group with a diameter of 100 to 300 nm and a third group with a diameter of about 500 to 2500 nm.[16] Getts et al.[22] used 500 nm polystyrene microparticles in their study. According to the studies[17,19], polystyrene particles were found to have a better poly disperse index and enable a better nanoparticle separation compared to the PLGA nanoparticles produced.

Briefly, MOG peptides used in the present study, are part of the myelin basic protein and are applied in the induction of the EAE model along with proteolipid protein (PLP) and myelin basic protein(MBP) peptides. EDCI was used for peptide binding.[8,9]

The reason for higher amount intravenous injection of $PLGA_{500}$-MOG_{35-55} nanoparticles in this study compared to similar studies[17,22] was the lower MOG_{35-55} -PLGA binding. The peptide binding method used in this study was strongly influenced by the electrical charge of the nanoparticles. Given that Getts et al had used polystyrene nanoparticles, the amount of polystyrene-peptide nanoparticles were half the amount used in the present study.[22]

The DTH response showed that the mice primed with $PLGA_{500}$-MOG_{35-55} had a lower ear thickness increase when challenged with MOG_{35-55} compared to the control group primed with $PLGA_{500}$-$OVA_{323-339}$ ($p \leq 0.0001$) (Figure 4a). This finding was consistent with the results obtained by Getts et al[22], which shows that cell-mediated immunity is decreased in mice challenged with PLP, confirming the induction of tolerance.

The splenocytes proliferation assay was used as an indicator of cellular immune function. The mice primed with $PLGA_{500}$-MOG_{35-55} one week before the development of the EAE showed a significantly lower proliferation compared to $PLGA_{500}$-$OVA_{323-339}$ (as control group), when challenged with MOG antigen ($p \leq 0.0001$). The analysis of tolerance in the peptide-induced EAE mice showed that $PLGA_{500}$-MOG_{35-55} microparticles injecting one week before the development of the EAE could delay the incidence of syndromes and reduce mean clinical score ($p \leq 0.05$) (Figure 5). This result was similar to Getts. et al. investigations which obtained same results when using 500 nm polystyrene particles.[22]

A parallel study conducted on the tolerogenic effect of PLGA nanoparticles showed that coating these nanoparticles with peptide P31 reduces the syndromes of autoimmune type I diabetes. Suchitra et al. created a covalent bond between PLGA nanoparticles and peptide P31 using EDCI and succeeded to reduce the clinical syndromes of autoimmune diabetes in the animal models.[25,26]

The results of the present study showed that when the mice are primed intravenously with $PLGA_{100}$-MOG_{35-55} microparticles, the effects of immune tolerance in the DTH test, splenocytes proliferation and the prevention of the clinical syndromes of EAE are not observed (Figure 4, 5).

In our study the effects of MOG-coupled nanoparticles administration route on the induction of tolerance was investigated. One group of the animals was therefore primed subcutaneously. Although differences were not statistically significant, the results showed that, when the mice are primed subcutaneously, not only the PLGA nanoparticles showed no tolerance

effects; but also they hasten the emergence of the disease symptoms through exacerbating the animal's immune response (p>0.17) (Figure 6). Maldonado et al. obtained similar results in their study.[27]

The process used for separating the nanoparticles by centrifugation and filtration was only partly successful and its separation ability was less than ideal, as shown in the electron microscopy images, particles smaller than 200 nm could also be observed among the 500-nm nanoparticles separated. The presences of these nanoparticles appear to have no effect on the induction of tolerance; however, further studies are required to ascertain this lack of effectiveness.

We used bicinchoninic acid method (BCA) to calculate the peptide-nanoparticle binding percentage due to the impossibility of using radioactive peptides. Although this method does not have the same sensitivity as the radioactive method, it is relatively sufficient for meeting the purposes of the present study.[17]

Given that, the size of the nanoparticles and their injection site can affect the type of immune response generated. The amount of peptide challenged along with the nanoparticles may contribute to the induction of tolerance, as the disease syndromes may be fully prevented through the bond's increased efficiency percentage and through the higher injection of nanoparticles containing peptides. Nevertheless, to ensure the safety of the intravenous challenge of higher amounts of the nanoparticles, the effect of microparticles larger than 1000 nm needs to be examined in a separate study.

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