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Original Article

Nasal Immunization by (PLGA) Nanospheres Encapsulated with Tetanus Toxoid and (CpG-ODN)

Mohsen Tafaghodi[a,b*], Seyed Abolghasem Sajadi Tabassi[a,d] and Mahmoud Reza Jaafari[a,c]

[a]*School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.*
[b]*Pharmaceutical Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.*
[c]*Biotechnology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.*
[d]*Pharmacological Research Center of Medicinal Plants, Mashhad University of Medical Sciences, Mashhad, Iran.*

Abstract

In induction of systemic and mucosal immunity, particulate antigens are more effective than soluble antigens possibly because they are more efficiently endocytosed by mucosal-associated lymphoid tissue (MALT) M cells. In this study, we determined the systemic and mucosal immune responses in rabbits following intranasal immunization of tetanus toxoid TT and CpG-ODN encapsulated within PLGA nanospheres. The mean diameter of (TT) and TT+CpG nanospheres were 753±193 and 684±324 nm, respectively. Encapsulation efficiency of TT and CpG-ODN was determined as 52±7.8% and 30.2±7.4%, respectively. The highest nasal lavage (sIgA) titers were observed in groups immunized with nanosphere formulations, while the IgG and antitoxin titers were suppressed by these formulations. CpG-ODN as an adjuvant could increase the serum IgG and antitoxin titers when co-administered with TT solution or co-encapsulated with TT in PLGA nanospheres, but failed to potentiate the IgA titers in nasal lavages. No hemolysis was occurred on incubation of PLGA nanospheres and human (RBCs). Also after nasal administration of plain nanospheres to human volunteers, no local irritation was seen. Intranasal administration of nanospheres encapsulated with vaccines showed to be an effective way for inducing mucosal sIgA immune responses, and CpG-ODN could increase the systemic immune responses.

Keywords: Nasal immunization; PLGA; CpG-ODN; Nanosphere; Tetanus toxoid.

Introduction

Biodegradable poly(D,L-lactide-co-glycolide) (PLGA) have been widely used as sutures, prostheses and drug carriers because of their non-toxic nature and adjustable biodegradation properties (1). PLGA microspheres and nanospheres are promising delivery systems for proteins, peptides and DNA vaccines (2). Earlier research in microsphere vaccine delivery was aimed at achieving controlled release of antigens for induction of long term antibody responses following a single-administration and thus eliminating the need for multiple immunizations (2). In addition, microspheres also enhance immunogenicity of the encapsulated protein antigens due to their particulate nature (3,4). Several studies have shown that microspheres made from PLGA polymers used to encapsulate bacterial toxoids and bacterial protein antigens can induce

* Corresponding author:
E-mail: tafaghodim@mums.ac.ir

protective and long lasting immune responses in small animal models (5). Both systemic and mucosal immune responses have been achieved after encapsulation of protein antigens in PLGA microspheres (6). Several nasal immunization studies have shown that particulated antigens, for example those encapsulated within biodegradable microspheres or liposomes, stimulate superior immunity to soluble immunogens (4,7,8).

Oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs are now recognized as a new class of adjuvants (9,10). These have sequences similar to unmethylated CpG dinucleotides of bacterial DNA. The innate immune system can recognize and differentiate these CpG ODN from the vertebrate's methylated DNA with the help of pattern recognition receptors to trigger an immune response against the perceived bacterial infection (11). CpG ODN affects various components of the immune system (12-14). In general, CpG ODN induces strong humoral and cellular immune responses with a bias towards a T helper type 1 (Th1) response (15). CpG ODN appear to be a promising class of adjuvants for a wide variety of vaccine candidates such as hepatitis viral surface antigen (16), inactivated influenza virus (17) and viral peptides (18).

Adjuvants can improve the immune responses by different mechanisms: some act as delivery systems which protect the immunogen from denaturation, help to target and increase antigen uptake by antigen-presenting cells (APCs) or by co-entrapment and co-presentation of antigen and another kinds of adjuvants to the same APCs (17). It has been shown that co-encapsulation of both CpG-ODN and antigens in the same delivery system or physical linkage of CpG-ODN to antigen highly increases the immune response (2,4).

In the present study, CpG ODN as an adjuvant for nasal immunization, was co-encapsulated with a model protein vaccine, tetanus toxoid (TT). TT is routinely used for immunization not only for humans but also for horses, sheeps, companion animals and other farm animals. Nanospheres encapsulated with TT, or both TT and CpG-ODN, were formulated characterized and their feasibility in inducing mucosal and systemic immune responses were evaluated.

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Materials

Phosphorothioate CpG oligodeoxynucleotide (#1826) was purchased from Operon Technologies Inc. (Alameda, CA). PLGA 50:50 co-polymer (inherent viscosity 0.17 dl/g in hexafluroisopropanol) was purchased from Birmingham Polymer Inc. (Birmingham, AL, USA). Polyvinyl alcohol (PVA) (87-89% hydrolyzed, 31000-50000 g/mol), dichloromethane, acetonitrile and Coomassie blue were purchased from Merck (Darmschtadt, Germany). Tetanus toxoid solution (2500 Lf/ml) and alum-adsorbed tetanus toxoid (50 Lf/ml) were from Razi vaccine and serum research institute (Hesarak, Iran). Anti-rabbit IgG and IgA were purchased from Sigma (Missuri, USA) and Bethyl Laboratories Inc. (Texas, USA), respectively.

White albino rabbits weighing 2-2.5 kg were provided by Pasteur Institute of Iran (Tehran, Iran).

Preparation of PLGA nanospheres encapsulated with TT and/or CpG ODN

Nanospheres were prepared using a W/O/W emulsion and solvent evaporation technique (19). Briefly, CpG ODN (75 µl, 4.4 µg/µl in Tris-EDTA (TE buffer 10 mM, pH 8.3) and TT (75 µl, 1200 Lf) solutions were mixed and emulsified with PLGA solution (600 µl, 33% w/v) in chloroform for 20 s using a microtip probe sonicator (MSE, England) in amplitude 18. Ice-water bath was used for prevention of temperature rise in sonication processes. The W/O emulsion was then combined with PVA solution (8 ml, 7.5% w/v in TE buffer) and sonicated (40 s) to form the W/O/W emulsion. The secondary emulsion was then added to PVA solution. The emulsion was further stirred for 2 h. Nanospheres were collected by centrifugation (20000 g, 15 min, 4 °C) washed twice with distilled water and then lyophilized.

Morphology and size analysis of PLGA nanospheres

Scanning electron microscope (Leo, Oxford, UK) was used for both studying the morphological features of nanospheres and analyzing the size

distribution. For the latter purpose, using the SEM micrographs, the diameter of 250 nanospheres was randomly determined.

Determination of the encapsulation efficiency of tetanus toxoid (TT) and CpG ODN in PLGA nanospheres

The TT content of PLGA nanospheres was determined using a two-step extraction method (2). Nanospheres were dissolved in Dichloromethane and precipitated TT was separated by centrifugation. The protein content was estimated using Bradford protein assay method.

Nanospheres containing only CpG ODN were similarly dissolved and the oligodeoxynucleotide was extracted in TE buffer (10 mM, pH 8.3). The amount of ODN was estimated spectrophotometrically based on absorbance at 260 nm (20).

Nasal immunization studies

Female albino rabbits weighing 2-2.5 kg (4 animals per group) were nasally administered with the following formulations in days 0, 14 and 28 of experiment:

1) Blank PLGA nanospheres

2) 40 Lf TT solution

3) 40 Lf TT+10 µg CpG-ODN both in solution

4) 40 Lf TT in nanospheres

5) 40 Lf TT+10 µg CpG-ODN both in nanospheres

6) 40 Lf TT in nanospheres+10 µg CpG-ODN solution

7) 10 Lf Alum-adsorbed TT (IM injection)

Animals were first injected with 40 mg/kg ketamine HCl to prevent the sneezing after administrations. Nanospheres suspensions and solutions (200 µl, 100 µl in each nostril) were administered using an automatic pipetter (Eppendorf).

Each animal was bled in days 21, 42 and 63. After the third bleeding, animal were sacrificed, trachea was cut and nasal cavity was washed with 10 ml sterile normal saline. Sera and nasal lavages of each group were frozen until immunological assays.

The research adhered to the Principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985).

Determination of serum Anti-TT IgG titers and nasal lavages anti-TT IgA titers

Anti-TT antibodies in the rabbit serum and nasal lavage were detected and quantified by end-point titration using an enzyme-linked immunosorbent assay (ELISA) (4).

Toxin neutralization (TN) test

For determination of serum anti-TT antitoxin titers, the TN test was performed at L+/100 and L+/1000 levels by the methods described elsewhere (4, 21). The L+/100 and L+/1000 dose of tetanus toxin are the minimum amounts of tetanus toxin, when mixed respectively with 0.01 and 0.001 antitoxin unit [AU] of standard tetanus antitoxin, kills 100% of mice in 4 days. Tetanus toxin was diluted to L+/100 or L+/1000 doses per ml. Various dilutions of standard tetanus antitoxin and serum samples were mixed with L+/100 or L+/1000 doses of toxin. The volume was made up to 1 ml with normal saline. The toxin-antitoxin or toxin-serum mixtures were incubated at room temperature for 1 hour. Each mixture was assayed by injecting 0.5 ml subcutaneously into 3 mice. Mice were observed for 5 days for tetanic symptoms and deaths. The titers of samples were calculated against the standards in terms of AU/ml.

Erythrocyte lysis test

The experiment was essentially performed as mentioned by Bjork and Edman (22). Human RBCs were suspended in Mc Ilvaine's buffer (citric acid, NaCl, Na_2HPO_4) pH 7. Two hundred µl of RBC suspension (12% hematocrit) was incubated with 200 µl of PLGA nanospheres suspension (containing 0.25, 0.5 or 1 mg nanospheres) for 30 min at 37 °C. The absorbance of the supernatant was recorded at 540 nm.

Local irritation studies in human volunteers

Ten mg of blank PLGA nanospheres were suspended in 100 µl PBS buffer and administered into the right nostril of 4 healthy volunteer and any symptoms of local irritations including sneezing, coughing, tearing, nasal stinging and burning was recorded in a one week follow up period (4).

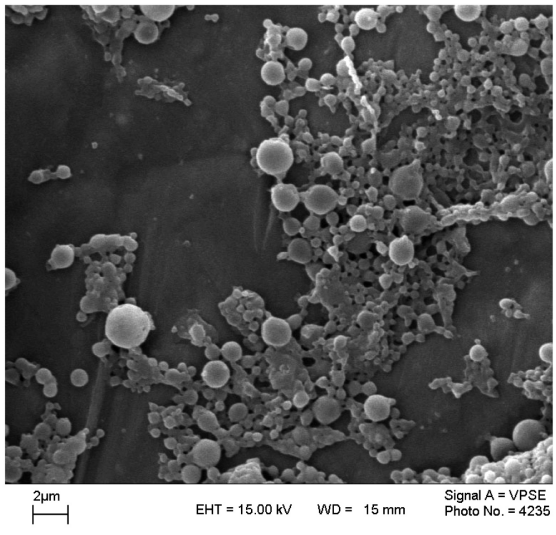

Figure 1. Scanning electron micrograph of PLGA nanospheres encapsulated with TT and CpG-ODN

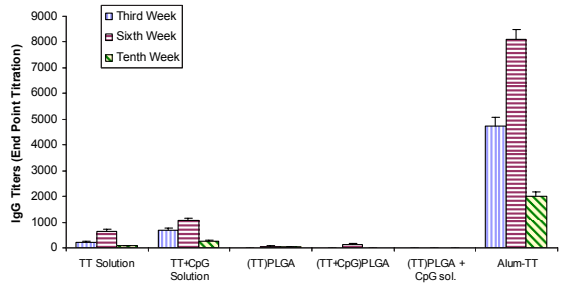

Figure 2. Serum anti-TT IgG titers. Rabbits (n = 4) were nasally immunized with 40 Lf TT and 10 µg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10 (10 Lf Alum-TT was injected intramuscularly as positive control). Sera anti-TT IgG titers (end point titration) were determined by an ELISA method. Error bars represent S.E.

This part of research followed the tenets of the Declaration of Helsinki promulgated in 1964 and was approved by the institutional human experimentation committee.

Statistical analysis

Statistical analysis was carried out by ANOVA and unpaired student t-test. P values less than 0.05 regarded as significant.

Results

Morphology and size of PLGA nanospheres

Mean diameters of PLGA nanospheres encapsulated with TT and TT+CpG ODN were respectively as 753±193 and 684±324 nm (n =250). The differences were not significant (P>0.05). The high SD values are mainly rooted from the preparation method (emulsification) rather than size analyzing method. Scanning electron micrograph of PLGA nanospheres encapsulating TT and CpG-ODN has been represented in Figure 1. As it is apparent, nanospheres were spherical in shape and had smooth surfaces.

Encapsulation efficiency of tetanus toxoid (TT) and CpG ODN in PLGA nanospheres

The encapsulation efficiency of tetanus toxoid (TT) and CpG ODN in PLGA nanospheres was determined to be 52±7.8% (n=3) and 30.2±7.4% (n=3), respectively. The loading of TT and CpG ODN was about 4 Lf /mg and 1 µg /mg nanospheres, respectively. Microsphere yields were determined as 85±3.2 (TT+CpG) and 91±4.3 (TT).

TT-release from microspheres

The toxoid was released from microspheres exhibiting a burst of 9.6±2.04% after 30 min incubation in the release media. This was followed by a slow and sustained release. After 30 days, a total of 20.5±0.76% toxoid release was recorded. The release of CpG ODN, a much smaller moiety (MW 6363) as compared to tetanus toxoid (MW 150 000 Da), was apparently faster. After 10 days of incubation, 54±2.6% cumulative release was measured.

Serum anti-TT IgG titers

Sera TT IgG titers were determined by ELISA (2). The highest IgG titers (Figure 2) among the nasally immunized animals were observed in groups immunized with TT and CpG solution (P<0.05). CpG-ODN as an immunopotentiating adjuvant could increase the serum IgG titers when co-administered with TT solution (P<0.05), or co-encapsulated with TT in PLGA nanospheres (P<0.05), but mixing of TT-PLGA nanospheres with CpG-ODN solution failed to potentiate the IgG response and even suppressed it (P<0.0001). Positive controls were intramuscularly injected with 10 Lf alum adsorbed TT and showed the highest IgG titers (P<0.0001). The sera IgG

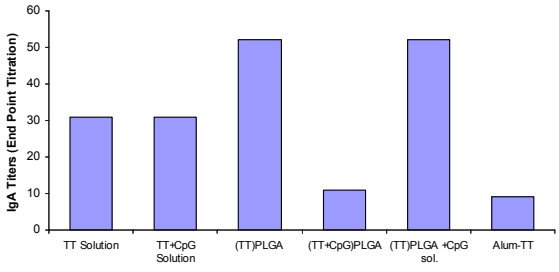

Figure 3. Nasal lavage anti-TT IgA titers. Rabbits (n = 4) were nasally immunized with 40 Lf TT and 10 µg CpG-ODN, at weeks 0, 2 and 4 and nasal lavages were collected at week 10 (10 Lf Alum-TT was injected intramuscularly as positive control). Lavages were pooled and Anti-TT IgA titers (end point titration) were determined by an ELISA method.

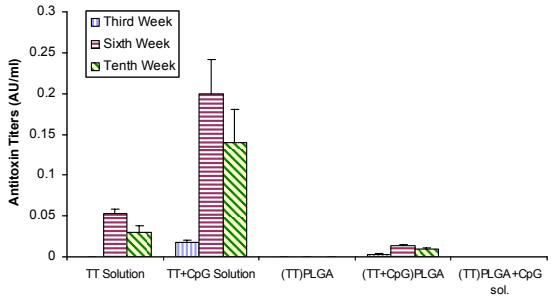

Figure 4. Serum anti-TT antitoxin titers. Rabbits (n = 4) were nasally immunized with 40 Lf TT and 10 µg CpG-ODN, at weeks 0, 2 and 4 and were bled at weeks 3, 6 and 10. Sera anti-TT antitoxin titers (AU/ml) were determined by toxin neutralization (TN) bioassay. Error bars represent S.E.

titers in animals nasally immunized with blank microspheres were not determined.

Nasal lavage anti-TT IgA titers

Among the groups immunized with various formulations, the highest mucosal IgA titers were seen in animals immunized with nanospheres encapsulated with TT (Figure 3). Co-encapsulation of CpG-ODN with TT in PLGA nanospheres failed to increase the IgA titers, and even suppressed the response. Similarly when CpG-ODN was simply mixed with TT solution no change was seen in the mucosal IgA titers. Intramuscular injection of alum-TT, resulted the lowest sIgA titers, compared with nasally immunized animals. The lavage sIgA titers in animals nasally immunized with blank microspheres were not determined.

Serum anti-TT antitoxin titers

The highest antitoxin titers were induced with nasal administration of TT and CpG-ODN solution ($P<0.05$) (Figure 4). The antitoxin titers induced by nanosphere formulations was lower than that of solutions ($P<0.05$). CpG-ODN as an immunomodulator adjuvant increased the antitoxin titers, both co-administered with TT solution or co-encapsulated with TT in PLGA nanospheres ($P< 0.05$). Nasal administration of blank PLGA nanospheres, as the negative control, resulted in zero antitoxin titer. Animals injected with 10 Lf alum-TT, as positive controls, showed higher antitoxin titers (4750, 8125 and 2000 IU/ml, in 3rd, 6th and 10th weeks) in comparison with nasally immunized animals ($P<0.0001$).

Hemolysis and nasal irritation

Different concentrations of nanospheres were incubated with erythrocyte suspension but no hemolysis was occurred.

PLGA nanosphere suspension was nasally administered to 4 human volunteers, but no irritation was reported. There was no report of sneezing, coughing, stinging or burning sensation in the nose, immediately after administration and also in one week following up.

Discussion

The results presented herein indicate that intranasal administration of PLGA nanospheres encapsulated with tetanus toxoid (TT) failed to induce systemic immune responses. Animals immunized with TT-containing PLGA nanospheres showed lower systemic ($P<0.001$) and higher mucosal immune responses, compared to liquid formulations. Nanospheres encapsulated with TT+CpG-ODN produced higher serum IgG ($P<0.05$) and antitoxin titers and similar nasal lavage IgA titers, compared with TT-nanospheres. When solution of CpG-ODN was mixed with TT-nanospheres, the lowest systemic and mucosal immune responses were achieved. In a previous study (2), mice were subcutaneously immunized with PLGA nanospheres encapsulated with TT or TT+CpG-ODN. Co-encapsulation of TT with CpG-ODN

in PLGA nanospheres was able to potentiate the systemic humoral and cellular immune responses (significantly higher IgG, IgG1, IgG2a, IgG2b and IFN-γ titers), when compared to TT-nanospheres and TT or TT+CpG solutions. However, mixing of TT-nanospheres with CpG-ODN solution, same as the present study, resulted in lower antibody titers than (TT) or (TT+CpG) nanospheres. In our another study with TT loaded liposomes, co-encapsulation of CpG-ODN could increase the serum IgG titers (7). It has also been shown that co-encapsulation of both CpG-ODN and antigens in the same delivery system or physical linkage of CpG-ODN to the antigen, highly increases the immune responses (23,24). These results indicated that PLGA nanospheres not only act as carrier system but also by co-encapsulation of antigen (TT) and adjuvant (CpG-ODN) and their co-delivery to the same APCs have an important role in the enhancement of immune responses (2).

Despite the expanding body of information concerning the uptake and distribution of microparticulate material following peroral administration, comparatively little is known about the fate of nasally delivered particulates (8). Some studies have demonstrated that nasally applied latex microspheres could rapidly enter the blood circulation, and hence access systemic immunoresponsive tissues in the spleen, indicating that microparticles are translocated through the nasal epithelium (25). Particles larger than 3 μm in diameter have been shown in humans to be retained in the nasal cavity when inhaled (26) and it has been observed in calves that tonsils could absorb resin particles of 1-5 μm in diameter (27). This absorption has been demonstrated in bovine pharyngeal tonsils to occur at the lymphoepithelium and the associated M cells (28). M-cells, thought to be the principal uptake site of particulate antigen, are found in the distal regions of the nose, the nasopharyngeal and palatine tonsils, and bronchial associated lymphoid tissues (BALT) in the lung (8).

It has been suggested that particle size may determine the type of the immune response elicited by vaccine-containing microspheres administered by the oral route. In this regard, microspheres less than 5 μm would be predicted to induce a predominantly circulating antibody response based on their propensity to disseminate to systemic lymphoid tissue. In contrast, microspheres greater than 5 μm would be predicted to raise predominantly a mucosal immune response because they remain in the IgA inductive environment of the Peyer's patches over the course of antigen release (29). In the present study, both (TT) and (TT+CpG) nanospheres could induce high mucosal immune responses. The same results were also obtained with alginate microspheres encapsulated with both TT and TT+CpG (4), and TT-loaded liposomes (7). But (TT) and (TT+CpG) PLGA nanospheres failed to potetiate the systemic immunity compared to TT and TT+CpG solution. Regards to the mean diameter of nanospheres (684±324 nm), the resulted responses (enhancement of mucosal responses and suppression of systemic responses) could be attributed to aggregation of nanospheres in suspending media and their failure in efficient interaction with M cells.

Other studies have shown that either a negative or a positive charge is better than a neutral charge in enhancing the interaction of microparticles with mucin and subsequent uptake by epithelial cells (30). Thus negatively charged PLGA microspheres could interact with mucosal surfaces more efficiently than neutral ones. Our previous gamma-scintigraphic study in human nose, has also shown that the PLGA microspheres have a high mucoadhesion potential. In that study we compared the clearance rate of alginate, PLGA and Sephadex microspheres from human nose and showed that the clearance half-life of PLGA microspheres in human nose is more than 4 hours (19).

Eyles et. al. (8) intranasally immunised groups of six mice with soluble or PLGA nanosphere encapsulated tetanus toxoid. Nasal instillation of nanospheres in 10 μl of buffer, generated statistically depressed (P<0.001) serum anti-toxoid IgG responses in comparison to animals immunised with 10 or 50 μl of soluble vaccine, or 50 μl of nanosphere suspension.

At the present study lack of membrane toxicity and local irritation of PLGA nanospheres in human nose was studied. Different concentrations of blank PLGA nanospheres were incubated with human RBCs and no hemolysis was observed. This could

be interpreted as a safety issue for PLGA nanospheres. Tolerability of nanospheres by the users is of high practical importance. Any local irritation aroused by nanospheres could result in sneezing and rhinorrhea; both of them could expel out the particles and decrease in drug delivery efficiency. Nasal application of blank PLGA nanospheres to 4 human volunteer didn't caused any local irritation. This finding could also demonstrate one safety aspect of PLGA nanospheres for practical use.

It is thought that soluble antigen is sampled by accessory cells in the pseudostratified respiratory epithelium. Thus in the case of vaccine formulations that consist of antigen(s) in solution, it is feasible that systemic responses may be generated by simple 'nose-drop' application (8). In the present study, administration of 40 Lf of TT solution as nasal drop, could induce systemic and mucosal immune responses. Regards to protective levels of tetanus antitoxin serum titers (0.01 AU/ml), administration of 40 Lf TT solution could result in protective levels of antitoxin. Co-administration of TT and CpG-ODN solution increased both serum IgG and antitoxin titers ($P<0.01$), but IgA titers in nasal lavages remained unchanged. In our previous study (2), s.c. injection of TT+CpG-ODN solution to mice induced higher humoral and cellular immune responses, compared to TT solution.

Intramuscular injection of alum-adsorbed TT which is the usual route of vaccination against tetanus, was also used as positive control. Rabbits immunized with alum-TT showed highest serum IgG and antitoxin titers ($P<0.001$), but as expected, parenteral immunization could not induce mucosal responses and the lowest IgA titers was seen in nasal lavages of this group (Figure 3).

Conclusion

We showed that a strong mucosal IgA response could be induced in rabbits with intranasal administration of PLGA nanospheres encapsulated with TT. CpG-ODN as an immunomodulating adjuvant increased both the serum IgG and antitoxin titers either mixed with TT solution or co-encapsulated in nanospheres. Lack of membrane toxicity, as studied by a standard hemolysis test, and local irritation of PLGA nanospheres in human nose was also indicated for the first time.

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