Archives of Razi Institute, Vol. 77, No. 6 (2022) 2259-2268

DOI: 10.22092/ARI.2022.360447.2583

Original Article

Nasal Administration of M2e/CpG-ODN Encapsulated in N-Trimethyl Chitosan (TMC) Significantly Increases Specific Immune Responses in a Mouse Model

Taghizadeh, M[1], Dabaghian, M[2] *

1. Medical Vaccine Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
2. Department of Biotechnology, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

Received 17 July 2022; Accepted 11 August 2022
Corresponding Author: m.dabaghian@rvsri.ac.ir

Abstract

The nasal passage is the primary entry point for many infectious agents. Therefore, nasal vaccines that can overcome the limitations associated with antigen uptake are likely to play an important role in protecting these infectious agents. Thus, adjuvants and antigen-carrying systems that can induce a suitable mucosal and systemic immune response against their accompanying antigens are highly important. In this study, synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODN) accompanied by the recombinant ectodomain of influenza M2 protein were encapsulated in N-trimethyl chitosan (TMC) nanoparticles. After the preparation of TMC nanoparticles, the morphological characteristics and loading efficiency and *in vitro* antigen release, as well as their ability to induce efficient immune responses against M2e in intranasal inoculation in the mouse model, were studied. Based on the size and zeta potential of the nanoparticles prepared in this study, it was determined that they were all nanosized, and their positive zeta potential ranged from 25 to 28 mV, while their polydispersity index was between 0.1 to 0.2, indicating a narrow range of particle sizes. A significant increase in serum levels of the total M2e-specific IgG antibody and BALF anti-M2e IgA was observed in mice intranasally immunized with M2e/CpG-ODN/TMC as opposed to those that were intranasally immunized with M2e/TMC, M2e/CpG-ODN, free M2e, and CpG-ODN/TMC. There was also a significant change in the IgG2a/IgG1 ratio in favour of IgG2a seems that CpG-ODN is responsible for directing the immune system towards Th1. Our findings show that CpG-ODN can significantly enhance the mucosal and systemic humoral immune response against M2e when encapsulated in a suitable carrier such as TMC for intranasal administration. In conclusion, when combined with a suitable carrier, CpG-ODN can be considered an effective adjuvant for mucosal administration.

Keywords: CpG-ODN 2006, M2e, TMC

1. Introduction

Intranasal vaccination is expected to promote better immune responses because the upper airway mucosal tissues are the primary entry point for the influenza virus and the mucosal immune system is the first line of defence against this pathogen (1). Hemagglutinin and neuraminidase are two of the main components of commercial influenza vaccines. Significant variability exists between the two types of proteins found in different species. These proteins protect against various influenza strains and types (2). As a result, it is critical to developing effective influenza vaccines to use proteins conserved in different viruses.

The extracellular sequence of the influenza virus M2 protein (M2e) contains 24 amino acids and has a high level of conservation and invariance among the different influenza virus strains (3). Several studies have been conducted on different adjuvants (3-7). Adjuvants play an essential role in stimulating immune responses against antigens. Among the adjuvants, those that stimulate the response of T lymphocytes and cellular immunity are particularly important (7).

In a similar manner to DNA motifs commonly found in bacteria, synthetic oligodeoxynucleotides containing CpG motifs (CpG-ODN) are capable of activating TLR9. It has been demonstrated that CpG ODNs can generate Th1-biased responses, making them a practical choice when mitigating potential Th2 and Th17-induced immune responses (8).

We attempted to encapsulate M2e and CpG-ODN in an efficacious antigen carrier that could be administered as intranasal mucosal vaccines. This was based on considering the benefits of mucosal vaccination.

Compared with other methods of vaccination, mucosal vaccination has some advantages, such as individual administration, less cost, reduced risk of needle stick contamination, and the ability to be administered to many people (9).

With the conventional methods of administering vaccines, such as intramuscular or subcutaneous injection, it is expected that if the vaccine is sufficiently compelling, serum IgG titers will increase. However, this increase is not generally accompanied by a strong local immune response and the production of secretory IgA (S-IgA) on mucosal surfaces (8). In addition, a significant antibody found on mucosal surfaces, the S-IgA, has a high potential for neutralizing both intracellular and extracellular influenza viruses (9). An interesting point to be made about S-IgA: it has been shown that anti-hemagglutinin (HA) IgA is more effective than serum IgG at inducing cross-immunity (10). However, to date, scant attention has been paid to the role of M2e-specific IgA in protecting the body against influenza infection. In addition to these cases, it is imperative to mention that the respiratory tract is also the place of entry for the influenza virus (11).

In the nasal mucosa, nasal-associated lymphoid tissue (NALT) regulates mucosal immunity, promoting systemic and local immune responses (8). As a component of mucosa-associated lymphoid tissue, the NALT contains lymphocytes, the follicle-associated epithelium (FAE), and epithelial microfold cells. Antigens and microorganisms that enter the body during inhalation must reach the sub-epithelial lymphoid tissues to be able to exert their immune effects locally and systemically.

Several factors, such as the epithelial barrier, cilia that clear mucus, and enzymatic and chemical degradation, hinder access to administered antigens to the submucosal areas of the skin and prevent an effective immune response (9). A common method of circumventing these limitations is encapsulating antigens in suitable carriers (10). Several characteristics make chitosan and its derivatives ideal for use as antigen carriers (11). Deacetylating chitin's N-terminus produces chitosan (CS), an amino polysaccharide that is biocompatible and not toxic. Chitin is a natural polymer that can be extracted from crustaceans' shells or fungi's mycelium (12).

Since chitosan is soluble in acidic pH and its solubility is low in neutral and alkaline pH, it is essential to note that the sub-epithelial space has a neutral pH. Therefore, the sub-epithelial area's physiological (neutral) pH may make chitosan unstable. The N, N, N-trimethyl chitosan (TMC) has been developed to solve this problem and improve chitosan's solubility. There is evidence that trimethyl chitosan (TMC) can induce a broad antibody response (13), expedite the maturation of dendritic cells (11), and be more soluble at physiological pH levels than chitosan (14).

Further, trimethyl chitosan (TMC) can bond with mucin on mucosal surfaces, which prolongs the time it takes for the encapsulated antigen to become established on mucosal surfaces. Generally, this property is known as the adhesive property of trimethyl chitosan (TMC) (15). Because of all these factors, TMC is widely used in the intranasal delivery of antigens, including tetanus toxin (16) and hepatitis B virus surface antigen (HBsAg) (17).

This study examined whether TMC nanoparticles could be used as a carrier system for intranasal delivery of M2e and CpG-ODN. Combining recombinant M2e and CpG-ODN with TMC nanoparticles (M2e/CpG-ODN/TMC) stimulated immune responses against M2e when administered intranasally to female mice (Balb/C).

2. Materials and Methods

2.1. Peptide

Synthetic M2e peptide (23 amino acids, 2 to 24, SLLTEVETPIRNEWGCRCNDSSD, synthesized by solid phase technology at the GL Biochem, China, was used in this study.

2.2. Preparation of Nanoparticles

Using an ionic gelation method, trimethyl chitosan (TMC) nanoparticles were prepared to contain high amounts of CpG-ODN, M2e, and M2e/CpG-ODN. A variety of concentrations (0.5, 1, 1.5, and 2 mg/ml) of trimethyl chitosan (donated by Dr. Maryam Iman from the Faculty of Medicine at Baqiyatullah University of Medical Sciences) were prepared. TPP solution (1 mg/ml) was added with recombinant M2e protein (30 µg per mouse) (10 mg/ml solution) or/and CpG-ODN 2006 (10µg per mouse) (InvivoGen). A drop-by-drop solution of M2e/TPP, CpG-ODN/TPP, or M2e/CpG-ODN/TPP was then added to trimethyl chitosan (TMC) solution at room temperature for an hour. Glycerin was added to a suspension of antigen-rich trimethyl chitosan (TMC) nanoparticles and centrifuged at 10,000 g for 15 minutes. Physicochemical and immunological analyses of antigen-rich nanoparticles were performed after removing the supernatant and resuspending the precipitates in PBS (phosphate-buffered saline).

2.3. Characterization

2.3.1. Morphology

The zeta potential and size of synthesized nanoparticles were determined by dynamic light scattering (DLS) (Table 1) (18).

2.3.2. Loading Efficacy

A difference in protein added to the loading solution and protein remaining in the supernatant was measured to determine the amount of immobilized protein in nanoparticles. The amount of M2e peptide in the supernatant was calculated using the Micro BCA protein assay kit (Thermo Fisher) (19). After centrifuging the nanoparticle suspension at 18,000 g for 20 minutes, the supernatant was separated from the nanoparticles. This formula was used to calculate the encapsulation efficiency (EE %) of N-TMC nanoparticles containing M2e:

%EE= ((total amount of antigen) - (free antigen in the supernatant))/ ((total amount of antigen))X 100

2.3.3. *in vitro* Release

Trimethyl chitosan (TMC) nanoparticles containing M2e or M2e/CpG-ODN were isolated after centrifugation at 10,000 g at four °C on a glycerol substrate for 15 min. Incubation was carried out at 37°C with the resulting solution after discarding the supernatant and re-dissolving the nanoparticles in 0.1 M PBS (buffered saline) (pH=7.4). A BCA (bicinchoninic acid) protein assay method was used to analyze the amount of protein released by nanoparticles after 0.5 ml of the suspension was removed at different time intervals and centrifuged at 18,000 g for 15 minutes. It was used to analyze the amount of protein in the supernatant solution. To determine the initial value equal to the same volume removed, PBS buffer (phosphate salt solution with buffering properties) was added to the solution. A sample containing uncharged trimethyl chitosan (TMC) nanoparticles were used as a control solution (Figure 1) (20).

Table 1. Characterization of antigen-loaded TMC nanoparticles

Groups	Average Size (nm)	Zeta potential (mV)	Polydispersity index	Encapsulation efficacy	TMC (mg/ml)	TPP(mg/ml)
M2e/CpG /TMC	356±18	25.3	0.133	94.71±1.6	1	1
M2e /TMC	214±12	28.2	0.142	95.2±1.8	1	1
CpG /TMC	321±12	26.2	0.182	93.52±2.2	1	1

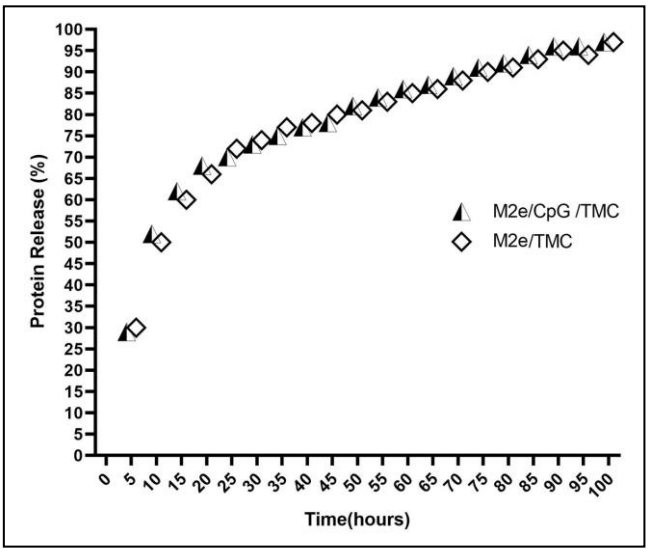

Figure 1. *in vitro* release profile of M2e from TMC nanoparticles

2.4. Immunization of Mice

From the laboratory animal department, 120 female mice aged 6 to 8 weeks were taken. During the experiment, mice were fed a standard diet.

To achieve the best results, the mice were divided randomly into several groups, each consisting of 20 mice, and the intranasal (i.n.) vaccinations were performed two weeks apart. To perform vaccinations, mice are anaesthetized intraperitoneally (IP) with ketamine (100 mg/kg) and xylazine (10 mg/kg). Each mouse received a total dose of 30 µg of M2e and 10 µg CpG-ODN, which was released in an equal volume of 5 µl per nostril with a micropipette tip (see Table 2).

Table 2. Experimental vaccination design, number of mice per group

Groups	Vaccination	Route of Immunization	Total number of mice
1	M2e/CpG-ODN/TMC	IN (Mice)	20
2	M2e/CpG-ODN	IN (Mice)	20
3	M2e/TMC	IN (Mice)	20
4	M2e	IN (Mice)	20
5	CpG-ODN/TMC	IN (Mice)	20
6	PBS	IN (Mice)	20

2.5. Serological Analysis

ELISA (enzyme-linked immunosorbent assay) technology was used to assess serum levels of anti-M2e IgG, IgG1, and IgG2a after each vaccination. We performed the ELISA test with some modifications described by Zhao, Lin (21). Briefly, wells of a 96-well plate (Maxisorb, Nank, Denmark) were coated with 100 µl of 1µg/ml synthesized M2e peptide (23 amino acids, 2-24, LLTEVETPIRNEWGCRCNDSSD, synthesized using solid phase technology, China at GL Biochem) were covered and incubated overnight in 50 mM sodium carbonate buffer with pH=6.9 at 4°C. The plates were washed with PBS (phosphate buffered saline) containing 0.05% polysorbate 20 Tween-20 (PBST), and then the wells were incubated with 100 µl blocking buffer (2% BSA in PBST) for 1 hour at 37°C. After three washes, serum samples (diluted in PBS at a ratio of 1:200) were placed on peptide-coated plates. The plates were incubated separately with different serum samples, and after washing three times with 1:10,000 anti-mouse IgG-HRP conjugate (Sigma Aldrich, USA), 1:4,000 anti-mouse IgG1-HRP conjugate (Southern Biotech, Birmingham, AL) or 1:4000 anti-mouse IgG2a-HRP conjugate (Southern Biotech, Birmingham, AL) were incubated for one hour at 37°C. After washing five times, the colour reaction was obtained with the help of 3, 3, 5, and 5-tetramethylbenzidine TMB (Pishtaztab, Tehran, Iran) at 450 nm.

Each serum sample was repeated in triplicate. Positive and negative control sera were included in each test. For comparison between groups, the mean values of different sera were analyzed at a wavelength of 450 nm.

2.5.1. Broncho Alveolar Lavage Fluids (BALF) IgA Assay

ELISA kits were used to determine IgA titers in bronchoalveolar lavage fluids (R&D, USA). The M2e peptide was used as a coating antigen for measuring IgA levels in the same way mentioned in the serum analysis section. To obtain bronchoalveolar lavage fluid, 1 ml of PBS was used to wash the trachea using a 25 mm catheter.

2.6. Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM) and represent at least three experiments

performed in triplicate wells. One-way analysis of variance (with SPSS 13.0 biostatistical software) was used to determine the significant difference between experimental groups. T-Student's test was used to determine the significant difference between the control and experimental groups. A p-value equal to 0.05 or less was determined as the significance level.

3. Results

3.1. Nanoparticle Formulation

The size and zeta potential of trimethyl chitosan (TMC) nanoparticles were analyzed using DLS and TEM techniques for the various concentrations of trimethyl chitosan (TMC) containing antigens (M2e/CpG-ODN//TMC, M2e/TMC and CpG-ODN/TMC nanoparticles) (Table 1).

Table 1 shows the results of measuring the size and zeta potential of the nanoparticles prepared in this research and the fact that they all have nano sizes, as seen from the measurements. Using trimethyl chitosan (TMC) at a concentration of 1 mg/ml, a polydispersity index of 0.2-0.1 indicates that the particles made are within a narrow size range.

There was a similar average diameter of M2e/CpG-ODN/TMC, CpG-ODN/TMC nanoparticles (between 320 and 360 nm, table 1) and 214±12 for M2e/TMC, a positive zeta potential of about +25-28 mV, and a PDI of about 0.13-0.18. Nanoparticles were used to measure the loading and release of antigens in the test tube and vaccinated mice. Based on the BCA (bicinchoninic acid) protein assay method, the encapsulation effectiveness of the nanoparticles was determined to be 94.71±1.6, 95.2±1.8 and 93.52±2.2, respectively for M2e/CpG-ODN/TMC, M2e/TMC and CpG-ODN/TMC (Table 1).

Then, the release rate of M2e from trimethyl chitosan (TMC) nanoparticles in PBS (phosphate salt solution with buffer properties) was investigated for 100 hours at 37°C and pH=7.4. Approximately 65% of the antigen was released during the first 12 hours, followed by an average of about 80% at 48 hours. After 100 hours, the percentage was 95% (Figure 1).

3.2. Humoral Immune Response following Immunization

The anti-M2e immune response was determined by detecting anti-M2e IgG titers in mouse sera following intranasal vaccination with M2e or/and CpG-ODN encapsulated in trimethyl chitosan (TMC) nanoparticles. Each vaccination was preceded by serum collection. Figure 2A shows that mice receiving M2e/CpG-ODN/TMC once had significantly higher anti-M2e IgG antibody levels than mice receiving other formulations (P<0.05). The anti-M2e IgG titer of mice vaccinated with M2e/CpG-ODN/TMC was statistically higher than that of other groups after the second vaccination (P<0.05). Only baseline anti-M2e responses were observed in the groups, including M2e and M2e/CpG-ODN.

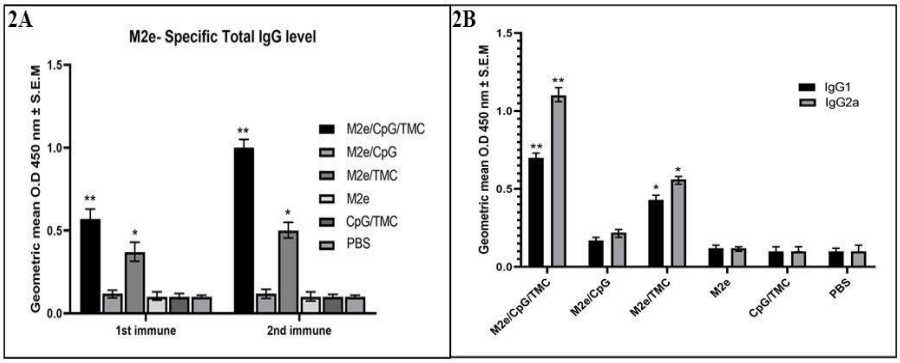

Figure 2. Anti-M2e total IgG response in mice nasally immunized with M2e/CpG/TMC, M2e/TMC, M2e/CpG, free M2e, CpG/TMC or PBS. By ELISA, sera were tested for (A) M2e-specific total IgG level or (B) M2e-specific IgG isotype antibody level two weeks after the first and second immunizations. The mean value ± S.E.M of A450 in each group was presented. The bars represent the mean value and standard error of the mean (S.E.M.) of twenty mice per group at each time point

Titers of antibodies were significantly higher than those obtained M2e/TMC. After the administration of M2e/TMC, the IgG2a to IgG1 was remarkably altered, resulting in an equal ratio of IgG2a to IgG1. As it is clear from the M2e/TMC group data, it seems that CpG-ODN is responsible for directing the immune system toward Th1. A small amount of anti-M2e IgG2a was detected in the sera of mice receiving the M2e vaccine, but no anti-M2e IgG1 antibody was detected. In the other groups, neither IgG2a nor IgG1 antibodies were detected against M2e.

3.3. Amount of IgA in Broncho Alveolar Lavage Fluid (BALF)

We calculated the amount of M2e-specific IgA in bronchoalveolar lavage fluid from different groups, as shown in figure 3. It was found that intranasal vaccination of mice using M2e/CpG-ODN/TMC resulted in a significant increase in IgA antibody levels in their bronchoalveolar lavage fluid compared with other vaccination approaches. Based on figure 3, anti-M2e IgA antibody titers were significantly increased in bronchoalveolar lavage fluid (BALF) of mice vaccinated with M2e/CpG-ODN/TMC. The other groups, on the other hand, did not develop significant amounts of specific IgA responses in comparison with the first group (Figure 3).

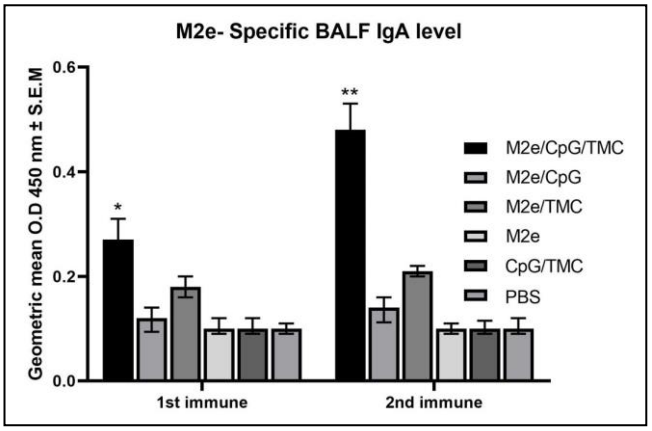

Figure 3. Determination of anti-M2e IgA in Broncho-alveolar lavage fluids (BALF) (N=5). S.E.M. (standard errors of the mean) are indicated by the error bars. Differences that are statistically significant (P<0.05) are indicated by (*) and (**)

4. Discussion

With intranasal vaccination offering unique advantages (23, 24), studies on improving the immune response to antigens administered this way could be extremely useful and essential (18, 25).

Therefore, we tested whether mice could be vaccinated against M2e using N-trimethyl chitosan (TMC) encapsulated with CpG-ODN administered through the nose in the present study. As part of the study, antibodies generated in response to M2e were measured for humoral immunity.

TMC nanoparticles carrying antigens were prepared using the ion gel method and crosslinked through the interaction between negatively charged tripolyphosphate (TPP) and positively charged amino trimethyl chitosan (TMC). Trimethyl chitosan (TMC) nanoparticles can be loaded with high levels of M2e and M2e with CpG-ODN (about 95%) using the ion gelation method.

The average size of the trimethyl chitosan (TMC) nanoparticles, which contained M2e and M2e/CpG-ODN, was 214 nm and 256 nm, respectively.

Several studies have been conducted on nasal vaccines (8, 25). M cells, epithelial cells, and dendritic cells take up particles of 1000 nm or less, better than particles of larger sizes (26). It has also been shown that there is probably no difference between the immunogenicity of 200 or 500 nm particles (27).

The surface charge of particles shows the stability of their formulation and is an essential parameter in determining the immunogenicity of antigens (28, 29).

The zeta potentials of M2e/CpG-ODN/TMC and M2e/TMC formulations, respectively, reached 25.3 mV and 28.2 mV based on the results of this research. As a result of electrostatic interactions between positively charged nanoparticles of trimethyl chitosan (TMC) and anionic glycoproteins on the mucosa, antigens are probably kept on the mucosa for more extended periods of time, and their absorption under the epithelium through M cells (22, 30).

Additionally, nanoparticles may bind to negatively charged cell membranes more readily due to their higher positive charge (31, 32).

Several factors influence the Th1/Th2 balance, including trimethyl chitosan (TMC), antigens, and immunological pathways (33, 34). A significant shift in IgG2a/IgG1 ratio was observed in mice incubated with M2e/CpG-ODN/TMC compared to M2e/TMC, suggesting a Th1-mediated immune response. As a result of these findings, CpG-ODN may play a crucial role in shifting immune responses toward Th1 (35, 36). The higher affinity of IgG2a for complement factors and Fc receptors in mice can explain why the Th1-mediated immune response against M2e provides better protection against lethal doses of influenza in mice (37, 38).

We examined serum and bronchoalveolar lavage fluid after mice were vaccinated with M2e/CpG-ODN/TMC and found that this vaccine can induce immune responses. A shallow level of immune response was induced when mice were incubated with free M2e/CpG-ODN.

According to this study, intranasal vaccination with free M2e/CpG-ODN or M2e cannot induce a significant immune response than intranasal vaccination with M2e/CpG-ODN/TMC, suggesting that mucociliary clearance reduces the residence time for non-mucoadhesive formulations in the nasal cavity. Accordingly, in accordance with a mucosal barrier, this feature hinders antigen uptake from mucosal surfaces, resulting in diminished vaccine efficacy (39, 40).

Therefore, efforts are being made to increase antigen persistence in the nasal cavity for longer. Furthermore, chitosan and TMC exhibit mucoadhesion properties and the ability to open tight epithelial junctions. Consequently, antigens are more accessible to immune induction sites due to increased paracellular transport (41).

Chitosan and its derivatives are commonly used as antigen carriers through the mucosal layer because of their ability to attach to mucus and open tight junctions (42, 43).

Furthermore, serum anti-M2e IgG results showed that, unlike M2e/CpG-ODN/TMC, free M2e did not induce significant serum IgG levels.

Based on previous findings, M2e remains poorly immunogenic per se, and suitable adjuvant formulations are needed to improve its immunogenicity (44).

Based on our study, the cooperation of both adjuvants, TMC and CpG-ODN, appears to be necessary to produce a significant immune response following intranasal administration of M2e.

To the best of our knowledge, no study has been conducted to test specifically the role that anti-M2e IgA plays in protecting mice against the influenza virus. In addition to serving as the significant antibody that covers the mucosal surfaces, IgA is secreted by plasma cells within the mucosa and is transported by epithelial cells through the proof of transcytosis (45).

Occasionally, transcytosis of IgA may protect epithelial cells infected with pathogens (46, 47). IgA could be alleviated by IgA, specifically for the newly synthesized M2 protein in infected epithelial cells.

In conclusion, based on our findings, nasal vaccination with the M2e/CpG-ODN antigen encapsulated in N-Trimethyl Chitosan (TMC) nanoparticles system resulted in a significantly higher M2e-specific humoral immune response in comparison with mice vaccinated intranasally with free M2e or M2e/CpG-ODN antigens.

Authors' Contribution

Study concept and design: M. D.
Acquisition of data: M. D.
Analysis and interpretation of data: M. T.
Drafting of the manuscript: M. T.
Critical revision of the manuscript for important intellectual content: M. D.
Statistical analysis: M. T.

Administrative, technical, and material support: M. D.

Ethics

The Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran ethics committee approved the study protocol.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Allen A, Leonard AJ, Sellers LA. The mucus barrier. Its role in gastroduodenal mucosal protection. J Clin Gastroenterol. 1988;10:93-8.
2. Brett IC, Johansson BE. Immunization against influenza A virus: comparison of conventional inactivated, live-attenuated and recombinant baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology. 2005;339(2):273-80.
3. Liu W, Zou P, Ding J, Lu Y, Chen Y-H. Sequence comparison between the extracellular domain of M2 protein human and avian influenza A virus provides new information for bivalent influenza vaccine design. Microbes Infect. 2005;7(2):171-7.
4. Bessa J, Schmitz N, Hinton HJ, Schwarz K, Jegerlehner A, Bachmann MF. Efficient induction of mucosal and systemic immune responses by virus-like particles administered intranasally: implications for vaccine design. Eur J Immunol. 2008;38(1):114-26.
5. Fan J, Liang X, Horton MS, Perry HC, Citron MP, Heidecker GJ, et al. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine. 2004;22(23-24):2993-3003.
6. Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine. 2008;26(2):201-14.
7. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol. 2004;82(5):488-96.
8. Slütter B, Jiskoot W. Dual role of CpG as immune modulator and physical crosslinker in ovalbumin loaded N-trimethyl chitosan (TMC) nanoparticles for nasal vaccination. J Control Release. 2010;148(1):117-21.
9. Lamichhane A, Azegami T, Kiyono H. The mucosal immune system for vaccine development. Vaccine. 2014;32(49):6711-23.
10. Gupta NK, Tomar P, Sharma V, Dixit VK. Development and characterization of chitosan coated poly-(ε-caprolactone) nanoparticulate system for effective immunization against influenza. Vaccine. 2011;29(48):9026-37.
11. Cox R, Brokstad K, Ogra P. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol. 2004;59(1):1-15.
12. Amidi M, Romeijn SG, Borchard G, Junginger HE, Hennink WE, Jiskoot W. Preparation and characterization of protein-loaded N-trimethyl chitosan nanoparticles as nasal delivery system. J Control Release. 2006;111(1-2):107-16.
13. Köping-Höggård M, Sánchez A, Alonso MJ. Nanoparticles as carriers for nasal vaccine delivery. Expert Rev Vaccines. 2005;4(2):185-96.
14. Smith A, Perelman M, Hinchcliffe M. Chitosan: a promising safe and immune-enhancing adjuvant for intranasal vaccines. Hum Vaccin Immunother. 2014;10(3):797-807.
15. Islam MA, Firdous J, Choi Y-J, Yun C-H, Cho C-S. Design and application of chitosan microspheres as oral and nasal vaccine carriers: an updated review. Int J Nanomedicine. 2012;7:6077.
16. Verheul RJ, Amidi M, van der Wal S, van Riet E, Jiskoot W, Hennink WE. Synthesis, characterization and in vitro biological properties of O-methyl free N, N, N-trimethylated chitosan. Biomaterials. 2008;29(27):3642-9.
17. Hu L, Sun Y, Wu Y. Advances in chitosan-based drug delivery vehicles. Nanoscale. 2013;5(8):3103-11.
18. Teng Z, Meng L-Y, Yang J-K, He Z, Chen X-G, Liu Y. Bridging nanoplatform and vaccine delivery, a landscape of strategy to enhance nasal immunity. J Control Release. 2022;351:456-75.
19. Smith Pe, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano M, et al. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985;150(1):76-85.
20. Chen F, Zhang Z-R, Huang Y. Evaluation and modification of N-trimethyl chitosan chloride nanoparticles as protein carriers. Int J Pharm. 2007;336(1):166-73.

21. Zhao G, Lin Y, Du L, Guan J, Sun S, Sui H, et al. An M2e-based multiple antigenic peptide vaccine protects mice from lethal challenge with divergent H5N1 influenza viruses. Virol J. 2010;7(1):1-8.
22. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 2006;6(2):148-58.
23. Hervé P-L, Raliou M, Bourdieu C, Dubuquoy C, Petit-Camurdan A, Bertho N, et al. A novel subnucleocapsid nanoplatform for mucosal vaccination against influenza virus that targets the ectodomain of matrix protein 2. J Virol. 2014;88(1):325-38.
24. Zeng W, Tan AC, Horrocks K, Jackson DC. A lipidated form of the extracellular domain of influenza M2 protein as a self-adjuvanting vaccine candidate. Vaccine. 2015;33(30):3526-32.
25. Dhama K, Dhawan M, Tiwari R, Emran TB, Mitra S, Rabaan AA, et al. COVID-19 intranasal vaccines: current progress, advantages, prospects, and challenges. Hum Vaccin Immunother. 2022:1-11.
26. Vila A, Sanchez A, Evora C, Soriano I, McCallion O, Alonso M. PLA-PEG particles as nasal protein carriers: the influence of the particle size. Int J Pharm. 2005;292(1-2):43-52.
27. Gutierro I, Hernandez R, Igartua M, Gascon A, Pedraz J. Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. Vaccine. 2002;21(1-2):67-77.
28. Joseph A, Itskovitz-Cooper N, Samira S, Flasterstein O, Eliyahu H, Simberg D, et al. A new intranasal influenza vaccine based on a novel polycationic lipid—ceramide carbamoyl-spermine (CCS): I. Immunogenicity and efficacy studies in mice. Vaccine. 2006;24(18):3990-4006.
29. Even-Or O, Joseph A, Itskovitz-Cooper N, Samira S, Rochlin E, Eliyahu H, et al. A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS). II. Studies in mice and ferrets and mechanism of adjuvanticity. Vaccine. 2011;29(13):2474-86.
30. Pardeshi CV, Belgamwar VS. Controlled synthesis of N, N, N-trimethyl chitosan for modulated bioadhesion and nasal membrane permeability. Int J Biol Macromol. 2016;82:933-44.
31. Behrens I, Pena AIV, Alonso MJ, Kissel T. Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport. Pharm Res. 2002;19(8):1185-93.
32. Sandri G, Bonferoni MC, Rossi S, Ferrari F, Gibin S, Zambito Y, et al. Nanoparticles based on N-trimethylchitosan: evaluation of absorption properties using in vitro (Caco-2 cells) and ex vivo (excised rat jejunum) models. Eur J Pharm Biopharm. 2007;65(1):68-77.
33. Slütter B, Plapied L, Fievez V, Sande MA, des Rieux A, Schneider Y-J, et al. Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination. J Control Release. 2009;138(2):113-21.
34. McNeela EA, Jabbal-Gill I, Illum L, Pizza M, Rappuoli R, Podda A, et al. Intranasal immunization with genetically detoxified diphtheria toxin induces T cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan. Vaccine. 2004;22(8):909-14.
35. Lin H-T, Chen C-C, Chiao D-J, Chang T-Y, Chen X-A, Young J-J, et al. Nanoparticular CpG-adjuvanted SARS-CoV-2 S1 protein elicits broadly neutralizing and Th1-biased immunoreactivity in mice. Int J Biol Macromol. 2021;193:1885-97.
36. Zhou J, Deng GM. The role of bacterial DNA containing CpG motifs in diseases. J Leukoc Biol. 2021;109(5):991-8.
37. Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, et al. Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol. 2006;13(9):981-90.
38. Schmitz N, Beerli RR, Bauer M, Jegerlehner A, Dietmeier K, Maudrich M, et al. Universal vaccine against influenza virus: linking TLR signaling to anti-viral protection. Eur J Immunol. 2012;42(4):863-9.
39. Slütter B, Hagenaars N, Jiskoot W. Rational design of nasal vaccines. J Drug Target. 2008;16(1):1-17.
40. Baker Jr JR, Farazuddin M, Wong PT, O'Konek J, Immunology C. The unfulfilled potential of mucosal immunization. J Allergy Clin Immunol. 2022;150(1):1-11.
41. Vllasaliu D, Exposito-Harris R, Heras A, Casettari L, Garnett M, Illum L, et al. Tight junction modulation by chitosan nanoparticles: comparison with chitosan solution. Int J Pharm. 2010;400(1-2):183-93.
42. Zhao X, Lu C, Yang S, Ni R, Peng T, Zhang J. Synthesis of N, N, N-trimethyl chitosan-based nanospheres for the prolonged release of curcumin. Food Hydrocoll Health. 2022;2:100092.

43. Zare S, Kabiri M, Amini Y, Najafi A, Mohammadpour F, Ayati SH, et al. Immunological Assessment of Chitosan or Trimethyl Chitosan-Coated PLGA Nanospheres Containing Fusion Antigen as the Novel Vaccine Candidates Against Tuberculosis. AAPS PharmSciTech. 2022;23(1):1-14.
44. Tan MP, Tan WS, Mohamed Alitheen NB, Yap WB. M2e-Based Influenza Vaccines with Nucleoprotein: A Review. Vaccines. 2021;9(7):739.
45. Macpherson A, McCoy K, Johansen F, Brandtzaeg P. The immune geography of IgA induction and function. Mucosal Immunol. 2008;1(1):11-22.
46. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science. 1996;272(5258):104-7.
47. Kaetzel CS, Robinson JK, Chintalacharuvu KR, Vaerman J-P, Lamm ME. The polymeric immunoglobulin receptor (secretory component) mediates transport of immune complexes across epithelial cells: a local defense function for IgA. Proc Natl Acad Sci U S A. 1991;88(19):8796-800.