



Research paper

Gastro-intestinal delivery of influenza subunit vaccine formulation adjuvanted with Gram-positive enhancer matrix (GEM) particles

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ABSTRACT

In this study, a liquid formulation of influenza subunit vaccine admixed with Gram-positive enhancer matrix (GEM) particles as adjuvant was delivered to upper and lower parts of intestinal tract. The aim was to determine the most effective immunization site in the intestines. Mice were vaccinated with a liquid formulation of GEM and influenza subunit vaccine orally and rectally. The oral administration of the vaccine with GEM particles induced a better systemic and mucosal immune response than oral (vaccine only) and rectal (with and without adjuvant) immunizations. Rectal administration elicited high IgG1 responses but little IgG2a, indicating a Th2 dominated immune response. In contrast, the oral immunization with GEM particles elicited a balanced IgG1 and IgG2a response. In conclusion, our results demonstrate that GEM-adjuvanted influenza vaccine should be targeted to the upper part of the intestinal tract.

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1. Introduction

Influenza is a viral infection with significant mortality and morbidity, especially in elderly and high-risk populations [1]. Vaccination against influenza is most efficient in controlling the influenza epidemics and pandemics [2]. Most of the current inactivated influenza vaccines are delivered via the parenteral route [3,4]. However, the parenteral route of administration has many drawbacks like pain at site of injection, lower compliance, needle stick injuries and expensive due to the need of medically trained personnel and stability requirements [5,6]. In order to overcome these drawbacks, mucosal immunization would be an attractive alternative. In past decades, intranasal inactivated influenza vaccines have shown some promising results, but due to the safety concerns of the used adjuvant systems, their development is hampered [7]. On the other hand, oral administration is still considered the most accepted and safe route of administration [5,8,9]. In addition, it is known that oral immunization can induce both systemic and mucosal immunity, whereas parenteral vaccines induce only a systemic immune response [10–14]. As shown before, induction of

sIgA antibody responses in the mucosal associated lymphoid tissue contributes to protection against influenza infection [3,15,16].

Oral delivery of influenza vaccines has been studied extensively. Animal studies show that oral delivery of influenza vaccine can protect mice from lethal challenge [17–19]. However, in clinical studies, only the generation of mucosal antibodies and low systemic immune response were found [20,21]. In these clinical studies, the dosing of the vaccine was frequent, which may have resulted in oral tolerance. Recently, much emphasis has been on two objectives to optimize the oral vaccine formulation: (1) decreasing the dose of the vaccine and (2) reducing the frequency of dosing. In order to achieve these two objectives, potent adjuvants are required, which can increase the immunogenicity of the vaccines. Thus, adjuvants are needed which can potentiate the efficacy of the vaccine and are safe to use in humans.

Gram-positive enhancer matrix (GEM) particles present a novel adjuvant produced from food-grade, non-colonizing *Lactococcus lactis* bacteria that are present in many dairy products used by humans. GEM particles are non-living bacteria-like particles with a diameter of about 1 µm. They represent spheres of peptidoglycan, deprived of all the surface proteins and intracellular content [22–27]. In addition, GEM particles are easy to produce compared to other adjuvants like bilosomes, conjugated liposomes and artificial biodegradable microparticles [11–13,18,19]. GEM particles bound to malaria antigen delivered via the oral route in rabbits were able to induce higher serum antibody titers than via other mucosal

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routes [25]. Furthermore, it was reported that GEM particles after oral administration in rabbits did not generate antibody responses against themselves. Furthermore, the preclinical results from the toxicity studies in rabbits have indicated no adverse effects of GEM particles. Thus, GEM particles are a safe and promising adjuvant for the oral immunization with influenza subunit vaccine.

It has been reported previously that there is some degree of compartmentalization in the gut mucosa, as the upper and the lower intestinal mucosa have different immune inductive sites [28,29]. Consequently, it is important to determine to which part of the intestinal tract the vaccine should be delivered to induce an optimal immune response. Therefore, in this study, we compared two different routes, i.e. oral and rectal delivery of liquid formulation of GEM particles admixed with influenza subunit vaccine.

2. Materials and methods

2.1. Materials

Influenza monovalent subunit vaccine of strain A/Hiroshima (H3N2) was kindly provided by Solvay Biologicals (Weesp, The Netherlands). The concentration of the haemagglutinin (HA) in the vaccine was determined using the single radial immunodiffusion assay as described in European Pharmacopoeia guidelines [30,31].

2.2. GEM preparation

GEM particles were prepared as described earlier [26]. Briefly, a culture of *L. lactis* was harvested and washed once with sterile distilled water. The cells were resuspended in 0.1 M hydrochloric acid (HCl) and placed in a hot water bath of 99 °C for 30 min. The acid and heat treatment kills the bacteria, degrades DNA and proteins, and generates so-called GEM particles. The GEM particles were pelleted and washed three times in PBS (pH 7) and finally resuspended in PBS and stored at –20 °C. The particles were counted using a cell counter. One unit (1 U) of GEM particles was defined as 2.5×10^9 non-living particles.

2.3. Immunization experiments

BALB/c mice (6–8 weeks) purchased from Harlan, Zeist, The Netherlands were used for all the immunization experiments. Animal experiments were evaluated and approved by the Committee for Animal Experimentation of the University of Groningen. Mice were immunized three times, i.e. on day 0, 14, and 28 via oral or rectal routes as described previously [10]. Briefly, for oral immunization, mice were administered 20 µg subunit vaccine with or without 1 unit GEM particles intragastrically in 200 µl of sodium bicarbonate solution (3.2% w/v). The oral administration was performed without anaesthesia using a stainless steel feeding needle. Rectal administration was performed by administering 100 µl of subunit vaccine (20 µg HA) with or without GEM particles directly into the colon. Rectal administration was performed under inhalation anaesthesia (isoflurane/O₂). Mice were put on a 45° platform upside-down, and dose was administered using a flexible Teflon feeding needle. After administration, the mice were kept on upside-down position for 10 min to prevent the leakage of dose. The mice were divided in four groups as follows: oral vaccine; $n = 7$, oral vaccine + GEM; $n = 6$, rectal vaccine; $n = 8$, and rectal vaccine + GEM; $n = 7$. All animals were killed on day 42.

Blood sampling was performed two times i.e. on day 28 before the third immunization via orbita puncture and on day 42 via cardiac puncture. Sera were obtained by centrifugation and stored at

–20 °C until further analysis. The intestinal and nasal washes were performed as described earlier [10].

2.4. ELISA

The ELISA was performed as described previously [22]. Briefly, the plates were incubated overnight (37 °C) with 200 ng of HA/well and blocked with 3% bovine serum albumin (Sigma). The plates were washed and incubated with serum (or nasal and intestinal lavages) in serial dilution for 1.5 h at 37 °C. Next, plates with serum samples were washed and incubated with horseradish peroxidase (HRP)-conjugated goat antibodies directed against mouse IgG, IgG1 and IgG2a (Southern Biotech, Birmingham, AL). For the intestinal and nasal washes, plates were incubated with HRP-conjugated goat antibodies directed against IgA. Finally, the substrate solution (0.02% 1,2-phenylenediamine-dihydrochloride in 50 mM phosphate buffer pH 5.6, containing 0.006% H₂O₂) was added, and plates were incubated in the dark for 30 min at room temperature. The reaction was stopped by addition of 2 M H₂SO₄, and absorbance at 490 nm was read with a Benchmark Microplate reader (BioRad, Hercules, CA). Titers reported are the reciprocal of the calculated sample dilution corresponding with an A₄₉₀ ≥ 0.2 after background correction.

2.5. HA inhibition assay

The haemagglutination inhibition (HI) titers in serum were determined as described previously [22]. Briefly, kaolin-adsorbed, heat-inactivated serum was transferred in duplicate to 96-well round-bottom plate and serially diluted twofold in PBS (pH 7.4). Next, 50 µl containing four haemagglutination units of inactivated A/Hiroshima virus was added to each well and incubated for 40 min at room temperature. Finally, 50 µl of 1% guinea pig red blood cells was added to each well and incubated for 2 h at room temperature. The highest serum dilution capable of preventing haemagglutination was scored as HI titer.

2.6. Statistical analysis

The statistical analyses were performed using a one-way ANOVA test and Bonferroni's correction for multiple comparisons. The results are presented as mean ± standard mean error unless indicated otherwise.

3. Results

3.1. Systemic immune responses

Mice were immunized with influenza subunit vaccine with and without GEM particles via the two different routes of administration, i.e. oral and rectal. HI titers were determined to compare the protective capacity of the immunizations. As shown in Fig. 1, the oral immunization with the HA + GEM vaccine induced significantly higher ($p < 0.05$) HI titers than oral immunization without GEM particles and rectal immunization with and without the GEM particles. Only oral immunization with HA + GEM vaccine induced titers over 40.

Antigen-specific IgG titers were determined after the first (day 28) and second (day 42) booster doses (Fig. 2). After the first booster immunization, oral HA + GEM induced significantly higher IgG titers than other immunizations regimens. All immunizations showed a further increase in IgG titers after the second booster dose. Rectal immunization with HA + GEM vaccine induced similar IgG titers compared to rectal and oral immunizations with the

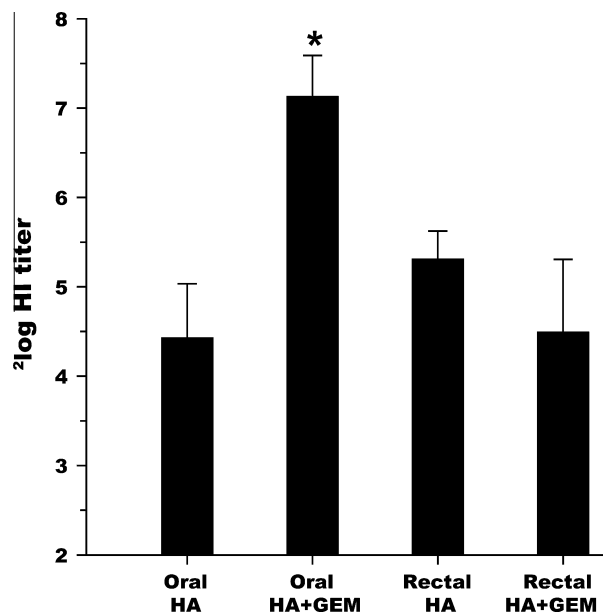


Fig. 1. Subunit antigen-specific HI titers in mice immunized with oral HA, oral HA + GEM, rectal HA and rectal HA + GEM influenza vaccine. * Indicates $p < 0.05$.

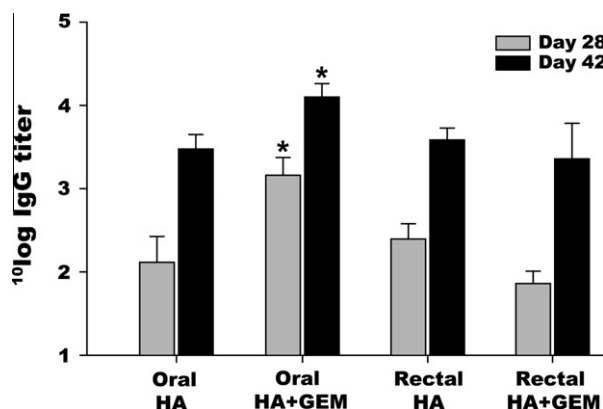


Fig. 2. Subunit antigen-specific total serum IgG titers in mice immunized with oral HA, oral HA + GEM, rectal HA and rectal HA + GEM influenza vaccine. The serum titers were determined at two time points, day 28 (grey bars) and day 42 (black bars). * Indicates $p < 0.05$.

influenza vaccine only. However, oral immunization with HA + GEM vaccine induced a significantly higher response.

3.2. Mucosal immune responses in intestinal and nasal washes

The mucosal immune responses were determined by measuring the sIgA titers in the intestinal (4 mice/group) and nasal washes (all mice). As shown in Fig. 3, the sIgA responses in the nasal and intestinal washes were low or absent in immunizations without the GEM particles. The oral immunizations with HA + GEM vaccine induced a better sIgA response in intestinal washes than rectal immunization with HA + GEM vaccine. Moreover, only oral immunization with HA + GEM vaccine was able to elicit substantial sIgA responses in nasal washes.

3.3. Phenotype of the immune response

The IgG subtypes (IgG1 and IgG2a) were determined to evaluate which type of T-helper cell response i.e. Th1 (high IgG2a levels) or

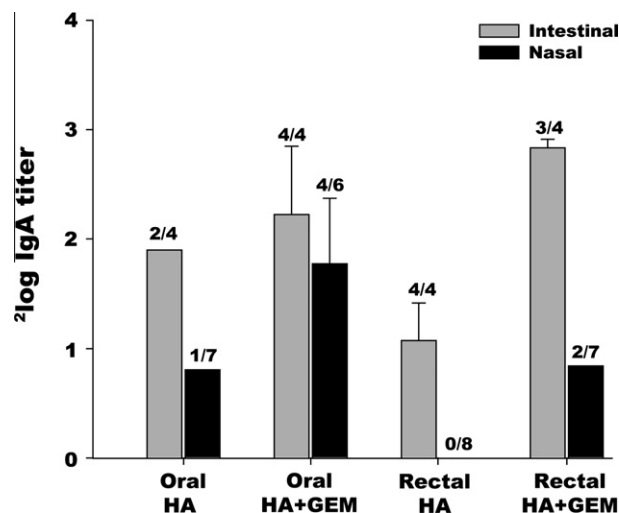


Fig. 3. Subunit antigen-specific sIgA titers in intestinal (grey bars) and nasal lavages (black bars) of mice immunized with oral HA, oral HA + GEM, rectal HA and rectal HA + GEM influenza vaccine. The numbers above the columns indicate the number of responders per group.

Th2 (high IgG1 levels) was elicited by the immunizations. Fig. 4 shows clearly that oral and rectal immunizations with influenza subunit vaccine only induced an IgG1 dominated response, indicating a dominant Th2-type response. Similarly, the HA + GEM rectal immunization induced a Th2-type response with dominant IgG1 levels. In contrast, the HA + GEM vaccine increased both the IgG1 and IgG2a responses when delivered orally, indicating a shift of immune response from a dominant Th2-type dominated response to a more balanced Th1/Th2-type response.

4. Discussion

In this study, we investigated the optimal target in the intestine for the delivery of the influenza vaccine adjuvanted with GEM particles. We showed that oral delivery of influenza subunit vaccine with GEM particles was able to elicit higher systemic immune response than non-adjuvanted and GEM-adjuvanted rectal vaccines. The oral HA + GEM vaccine induced three times higher HI titers than non-adjuvanted vaccines. HI titers in serum are used as a correlate of protection for influenza vaccines [32]. Only the oral vaccine adjuvanted with GEM particles was able to generate significantly high titers. In addition, this vaccine elicited higher serum IgG titers than non-adjuvanted oral, rectal and rectal GEM-adjuvanted vaccines. The results clearly indicate that GEM particles administered via the oral route enhance the immunogenicity of the oral influenza subunit vaccine and are capable of inducing high HI titers.

Antigens delivered via the oral route are processed by the gut-associated lymphoid tissue (GALT) in the digestive tract [9]. Usually in the upper intestine, the antigens can be processed via two different pathways, (A) by M cells present in the Peyer's patches which are located in the sub-mucosal layer of lamina propria [33] and (B) by the interstitially located dendritic cells in the epithelial lining of the intestine [34]. In case of the GEM particles, it has been found that GEM particles can be taken up by M cells and activate macrophages and dendritic cells [26,27,35]. This activation is caused by binding to Toll-like receptors (TLR)-2 [36]. Thus, the GEM particles are likely to be processed by both pathways and activate B and T cells. In our study, we mixed the GEM particles with influenza subunit vaccine and showed that when administered orally the mixture induced mucosal sIgA responses

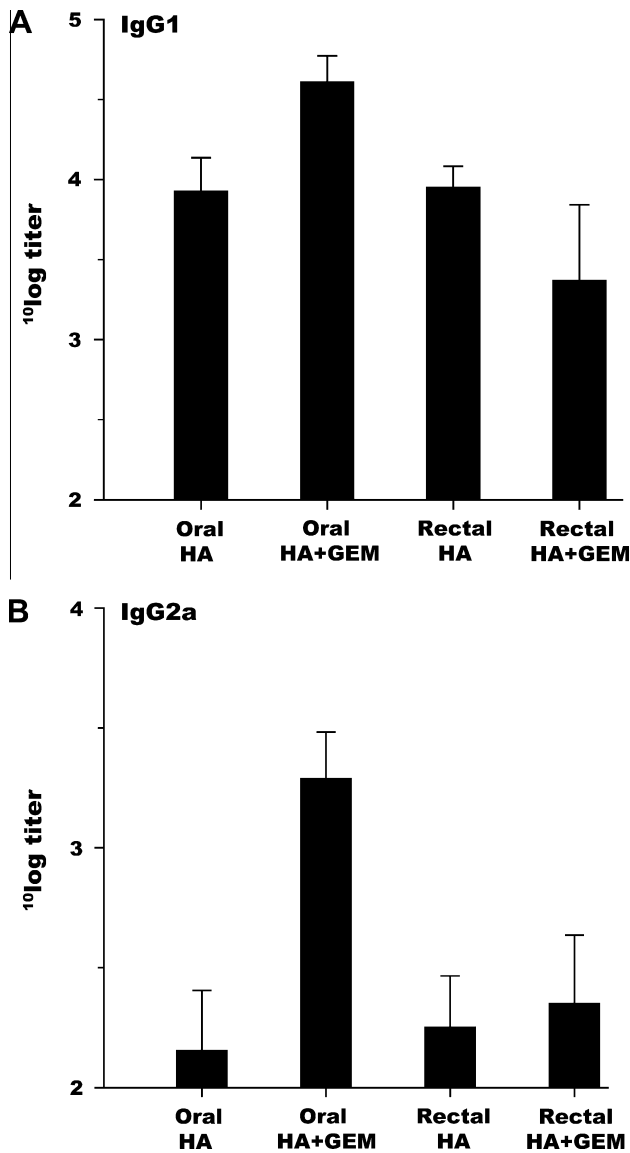


Fig. 4. Subunit antigen-specific serum IgG1 (A) and IgG2a (B) subtype titers in sera of mice immunized with oral HA, oral HA + GEM, rectal HA and rectal HA + GEM influenza vaccine.

in the intestinal tissue. In addition, the oral GEM-adjuvanted influenza subunit vaccine was also able to evoke the sIgA response in the nasal tissue. It was shown previously that migration of activated antigen presenting cells to distant mucosal site by lymphatics can result in induction of the sIgA in the nasal tissue [9]. The induction of the mucosal immune response in nasal tissue is important because it is the port of entry of influenza virus. However, the rectal delivery of the vaccines was not able to induce a strong nasal immune response. In the lower intestine, the antigens are processed by the lymphoid follicles [28,29]. It can be speculated from the results that the presentation of antigen and adjuvant is not optimal for uptake by lymphoid follicles. This might have resulted in sub-optimal stimulation of immune system by rectal administration of GEM and influenza subunit vaccine.

The available inactivated influenza vaccines adjuvanted with alum induce a Th2-type immune response [37]. However, the oral HA + GEM vaccine was able to increase IgG2a response, indicating a shift of immune response from Th2 dominated to a more balanced Th1/Th2 response. Recent findings with pandemic H1N1 whole virus vaccine suggest that a balanced Th1/Th2 response is

more protective [38]. In addition, the nature of the GEM particles such as their size (approximately 1 μ m) may have influenced the type of immune response, as shown in other studies [39]. Our present oral immunization studies with GEM particles seem to corroborate these findings.

In this study, the vaccine was delivered via the oral route as a liquid formulation in combination with sodium bicarbonate, which neutralizes the gastric pH. Obviously, this approach is not well suited for the clinical application. In general, enteric coatings are used to protect tablets or capsules from the acidic pH of the stomach [40]. For the development of a tablet formulation, both the influenza subunit vaccine and GEM particles need to be in a dried formulation. It has been shown previously that both GEM and influenza subunit vaccine can be dried without the loss of their structural and functional integrity [26,41]. In addition, previously our research group showed that an acid-sensitive enzyme alkaline phosphatase can be delivered to the intestine using enteric-coated tablets [42]. This indicates that in future an enteric-coated tablet formulation for intestinal release, containing the vaccine and GEM particles, can be developed.

5. Conclusions

In this study, we investigated the optimal target in the intestine for the delivery of the influenza subunit vaccine adjuvanted with GEM particles. Our data show that oral influenza subunit vaccine adjuvanted with GEM particles, delivered to the upper part of the intestine, induced systemic and mucosal antibody responses.

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