Potential of Medium-Chain Glycerides-Based Microemulsions for Provoking Immune Response Following Oral Administration

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Abstract

Medium-chain glycerides-based microemulsions have been demonstrated to enhance the intestinal absorption of some peptides. Thus, this may be beneficial for using as an antigen delivery system. Therefore, the present study was aimed to investigate the potential of a medium-chain glycerides-based w/o microemulsion for provoking immune response following oral administration using Balb/c mouse as a model. The microemulsion was prepared by mixing 76% of oil mixture (Crodamol GTCC:Capmul MCM 3:1), 14% of surfactant mixture (polysorbate 80:sorbitan mono-oleate, 3:2) and 10% water. Model antigen, ovalbumin (OVA), 50 mg was dissolved in 1 ml of the aqueous fraction of the microemulsion. The ability to elicit immune responses of the microemulsion containing OVA was investigated by administering orally for three consecutive days at four weeks after the mice were primed subcutaneously with nanoparticles containing OVA. The results were demonstrated that the medium-chain glycerides-based microemulsion provoked higher immune responses, compared to OVA solution, suggesting that the microemulsion prepared may be a useful delivery system for an antigen. However, the responses were not maintained for long period.
Keywords: microemulsions, immune, oral

Introduction

Microemulsions are thermodynamically stable, isotropically transparent (or translucent) dispersions of oil and water stabilized by interfacial films of surfactant and co-surfactant molecules (Eccleston, 1992). Microemulsions offer greater advantages over the conventional dispersion (i.e. suspensions and emulsions) and micellar solution (Ritschel, 1991; Constantinides, 1995; Gao *et al.*, 1998). They form spontaneously with the need of only mild agitation. They are thermodynamically stable leading to a long shelf-life (Eccleston, 1992). In addition, the order of mixing of the components does not have any effect on the characteristic of the final product and thus can easily be produced in a large scale (Constantinides, 1995).

Recently microemulsions has gained attention as a new drug delivery system for labile (proteins and peptides) and poorly soluble drug since they can be easily prepared as discussed above. Moreover, the preparation of microemulsions does not require high temperature and/or homogenization, which may affect the stability of the labile drugs. As a peroral delivery system, microemulsions offer several advantages including improvement of drug solubilization and protection of drug against enzymatic hydrolysis (Ritschel,

1991; Constantinides, 1995; Gao *et al.*, 1998). Moreover, the incorporation of more than one drug can be done in microemulsion system. It has also been claimed that the surfactant in the lipid-based delivery system including microemulsions can increase intestinal absorption by altering mucosal membrane fluidity, resulting in permeability changes (Swenson and Curatolo, 1992; Yeh *et al.*, 1994). Its potential for enhancing the delivery of drugs, proteins and peptides via oral route has been extensively demonstrated (Ritschel *et al.*, 1990; Ritschel, 1991; Ho *et al.*, 1996; Tenjarla, 1999; Gershanik and Benita, 2000). For example, Ritschel *et al.* (1990) reported that the absorption of cyclosporin A, a small peptide, formulated in a microemulsion was shown to be improved. They also demonstrated that the absorption of vasopressin in rats from ligated small intestinal segments was increased by two-fold when the peptide was formulated in a microemulsion as compared to an aqueous solution (Ritschel, 1991).

Microemulsions containing medium-chain glycerides derived from coconut oil were employed in this study. Since the medium-chain glycerides are naturally derived, they are considered as safe by the US Food and Drug Administration agency (Ritschel *et al.*, 1990; Ritschel, 1991). In addition, they were reported to improve the intestinal absorption of co-formulated drug (Ritschel, 1991; Charman *et al.*, 1992; Constantinides *et al.*, 1994; Constantinides, 1995). Constantinides *et al.* found that the water-in-oil (w/o) microemulsions particularly those contain medium-chain glycerides (mono-/di- and triglycerides) enhanced intestinal absorption of calcein (Constantinides *et al.*, 1994; Constantinides *et al.*, 1996) and RGD peptide, a cyclic tetrapeptide act as fibrinogen receptor antagonist SK&F 106760, in rats (Constantinides *et al.*, 1994). The mechanism of medium-chain glycerides to enhance absorption is involved with both transcellular and paracellular pathway (Constantinides *et al.*, 1996).

Following the absorption of antigen from small intestine, the effective immune responses will be initiated when the antigen is taken up through M-cells of the Peyer's patches (PPs). PPs are the lymphoid tissues where immunocompetent cells including B cells, T cells, macrophages and dendritic cells are present and the immune responses are initiated. As detailed above, microemulsions were shown to enhance the intestinal absorption of labile and poorly soluble drug and medium-chain glycerides have been reported to enhance intestinal absorption. Thus a medium-chain glycerides-based microemulsion may enhance the absorption of antigen leading to a high immune response. Therefore, the aim of the present study was to investigate the potential of a medium-chain glycerides-based w/o microemulsion for provoking immune response following oral administration using Balb/c mouse as a model.

Materials and methods

Materials

Caprylic/capric triglycerides (Crodamol GTCC®), polysorbate 80 (Crillet 4®) and sorbitan mono-oleate (Crill 4®) were obtained from BTB Chemicals Ltd (Auckland, NZ). Caprylic/capric mono-/diglycerides (Capmul MCM®) was obtained from Abitec Corp. (Columbus, OH, USA). Ovalbumin (grade V), monoclonal anti-chicken egg albumin (mAb to OVA), IgA kappa, goat anti-mouse IgA (α-chain specific) and rabbit anti-goat IgG peroxidase conjugate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2, 2, 2-tribromoethanol (Avertin®) and tert-amyl alcohol were supplied by Aldrich (St. Louis, MO, USA). Goat anti-mouse IgG horse radish peroxidase (HRP) conjugate ZyMax™ Grade and 3, 3′, 5, 5′ – tetramethylbenzidine (TMB) were supplied by Zymed Laboratories (San Diego, CA, USA). Tween 20 was obtained from Serva Electrophoresis GmbH (Heidelberg, Germany). Foetal calf serum (FCS, New Zealand origin) was purchased from Gibco BRL (Life Technologies, Melbourne, Australia).

Methods

Preparation of microemulsions

Medium-chain glycerides-based microemulsions employed in this study were chosen from the microemulsion area in the pseudoternary phase diagram constructed by Watnasirichaikul (Watnasirichaikul, 2000). The microemulsion containing 76% of oil mixture (Crodamol GTCC:Capmul MCM 3:1), 14% of surfactant mixture (polysorbate 80:sorbitan mono-oleate, 3:2) and 10% phosphate buffer solution (pH 7.4) as shown in Figure 1 was selected because it has low amount of surfactant and suitable viscosity (39 mPas as measured using Brookfield DVIII viscometer fitted with a CP-42 cone and plate spindle). Model antigen, i.e. ovalbumin (OVA) 50 mg/ml, was dissolved in aqueous fraction prior to mixing with oils/surfactants blend under magnetic stirring.

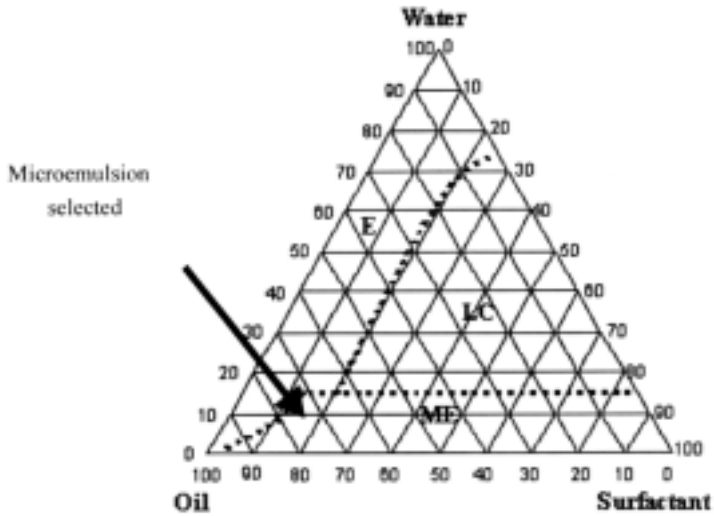

Figure 1. Pseudo-ternary phase diagram for a mixture of medium chain glycerides (Crodamol GTCC:Capmul MCM, 3:1), a mixture of surfactants (polysorbate 80:sorbitan mono-oleate, 3:2) and water (ME: microemulsion, LC: systems containing liquid crystals and E: coarse emulsions) (Watnasirichaikul 2000).

Preparation of poly(ethyl cyanoacrylate) nanoparticles containing OVA

Poly(ethyl cyanoacrylate) nanoparticles containing OVA were prepared as described previously (Pitaksuteepong, 2002; Pitaksuteepong *et al.*, 2002). Shortly, a microemulsion (10 g) containing OVA 50 mg/ml of the aqueous fraction was prepared as described above. The microemulsion was stirred at 4 °C prior to the addition of ethyl cyanoacrylate monomer (600 mg) dissolved in chloroform (ratio 1:3 by weight). The system was left at 4 °C overnight for polymerization before separating by centrifugation at 51,000 g for 60 min at 25 °C (Beckman J2/MC Centrifuge, JA 20.1 rotor, USA). The nanoparticles were then washed twice in pure ethanol and after each wash they were separated by centrifugation at 18,500 g for 10 min at 25 °C (Biofuge15, Heraeus Sepatech GmbH, Germany). Subsequently, the nanoparticles were redispersed in glucose solution 5% (w/v) in milli-Q water to obtain a final polymer concentration of 5 mg/ml. In each step of washing, the nanoparticles were redispersed using a bath sonicator. The resulting nanoparticle suspension in 5% glucose (4 ml) was placed in a 20 ml glass vial and frozen at -84 °C for 1 h before freeze drying for 2 days (-84 °C, 55 x 10^{-3} mbar) using a FreeZone® Plus 6 (Labconco, USA). The Freeze-dried nanoparticles were kept in a dessicator at 4 °C until used. The particles size of the prepared nanoparticles containing OVA was measured using photon correlation spectroscopy (Zetasizer 3000, Malvern Intruments Ltd., UK) and it was shown to be about 272.0 ± 18.5 nm. Loading content was 0.065 ± 0.007 mg per mg nanoparticles (Pitaksuteepong, 2002).

Animal model

Sixteen female BALB/c mice age 7-9 weeks were obtained from the Department of Laboratory Animal Sciences, University of Otago, New Zealand and reserved in a conventional facility. The study was approved by the Animal Ethics Committee, University of Otago, New Zealand (approval number 94/01).

Immunization protocols

Mice were divided into 2 groups each group comprising 8 mice. The mice were primed by subcutaneous injection into the dorsum (back) of the neck with 100 μg of OVA encapsulated in freezed-dried nanoparticles using ethylcyanoacrylate monomer 6% w/w. The nanoparticles were reconstituted with the appropriate volume of saline solution before injection.

At week 4, one group of the mice was boosted with OVA in saline solution whereas the other group was boosted with OVA in microemulsion. The booster doses were given orally using a blunt-tipped feeding needle for three consecutive days with 0.5 mg (volume 100 μl) of OVA formulated in either saline solution (control) or microemulsion.

Determination of immune responses

Immune responses to OVA dissolved in saline solution and in microemulsion were determined by measuring OVA-specific serum immunoglobulin G (IgG) and secretory immunoglobulin A (sIgA) antibody.

a) Determination of OVA-specific serum IgG antibody

Blood samples were collected from the cut tail tip of mice at 4 and 7 weeks following priming. At the end of the study (week 11), blood was collected via cardiac puncture following anaesthesia with an intraperitoneal injection of 125 mg/kg of 2,2,2 – tribromoethanol (Avertin®) dissolved in tert-amyl alcohol. The blood samples were allowed to clot overnight and then centrifuged at 8,000 g for 5 min at room temperature. For tail bleeds, serum was collected and pooled for each group of mice. For blood collected by cardiac puncture, serum from each mouse was kept separately. All serum samples were stored frozen at –20 °C until assayed.

The OVA-specific serum IgG antibody titer in serum sample was measured by ELISA as previously described (Pitaksuteepong, 2002). Briefly, 96-well MaxiSorp NUNC-Immuno™ plates, flat bottom (NUNC, Denmark) were coated with 50 μl/well of 100μg/ml OVA in coating solution (0.1 M $NaHCO_3$, pH 8.2). After overnight incubation at 4 °C, the plates were washed 6 times with 0.05% (v/v) tween 20 in phosphate buffer saline solution (T20/PBS). Blocking was carried out by adding 200 μl of 10% (v/v) FCS in PBS (10FCS/PBS) into the wells followed by a 2 h incubation at room temperature.

The plates were then washed 6 times with T20/PBS. One hundred μl of the serum was added to each well in duplicate. Two fold serial dilutions of the samples with 10FCS/PBS were carried out in the ELISA plates. One hundred μl of OVA specific monoclonal antibody at a concentration 100 ng/ml was diluted with 10FCS/PBS in the ELISA plates, resulting in a series of concentration ranging from 100 – 1.56 ng/ml (n = 2). Blanks were also set up in duplicate using 100 μl of 10FCS/PBS and the absorbance of these blanks was subtracted from the absorbance of the standards and samples. The ELISA plates were incubated for 1 h at room temperature and then washed 6 times with T20/PBS. Goat anti-mouse IgG HRP conjugate was diluted 1:5000 with 10FCS/PBS and 100 μl of the diluted solution added into each well and further incubated for 45 min at room temperature. The plates were washed 6 times with T20/PBS and 100 μl of 3, 3′, 5, 5′ – tetramethylbenzidine (TMB) was then added into each well. Following color development, the reaction was stopped by adding 100 μl/well of 1N H_2SO_4. The absorbance was measured at wavelength 450 nm using a Bio-Rad 550 Microplate reader (Bio-Rad Laboratories, CA, USA).

b) Determination of secretory IgA (sIgA) antibody

Six to eight pieces of freshly faecal samples from mice were collected at the same time as blood samples. The samples were either kept at–20 °C until assayed or subjected to vacuum drying using a Speed Vac Concentrator (SVC-200H) and Savant -Refrigerated Vapor Trap (RVT 4104) (Savant Instrument, Inc., USA). Secretory IgA was extracted from the faecal samples by adding phosphate-buffered saline solution (PBS) at a ratio of 15 μl per mg dry faeces. The solid faecal samples were then suspended by extensive vortexing. Subsequently, the suspensions were centrifuged at 12,000 g for 20 min. The clear supernatants were assayed for sIgA by ELISA using 96-well MaxiSorp NUNC-Immuno™ plates, flat bottom (NUNC, Denmark). The plates were coated with 50 μl/well of 250ng/ml IgA kappa and 100 μg/ml of OVA solution for standard and sample wells, respectively, in coating. For standard wells, doubling dilution in ELISA plate was performed to give IgA standard, i.e IgA kappa, with concentration ranging from 250 to 3.9 ng/ml). Blanks were also set up in duplicate using 100 μl of 10FCS/PBS. Following overnight incubation at 4 °C, the plates were washed 6 times with T20/PBS. Non-specific protein-binding sites were blocked by adding 200 μl of 10FCS/PBS into the wells followed by a 2 h incubation at room temperature. The plates were then washed 6 times with T20/PBS. One hundred μl of the sample was added to each of the sample well in duplicate while 100μl/ well of 10FCS/PBS was added into the wells set as standard and blank. The ELISA plates were incubated for 2 h at room temperature and then washed 6 times with T20/PBS. Goat anti-mouse IgA was diluted 1:2000 with 10FCS/PBS and 100 μl of the

diluted solution added into each well and further incubated for 30 min at room temperature. Then, 100 µl of rabbit anti-goat IgG peroxidase conjugate diluted 1:2000 with 10FCS/PBS was added into each well. After 15-min incubation at room temperature, the plates were washed 6 times. Subsequently, 100 µl of TMB was added into each well and allowed the color to develop. The reaction was stopped by adding 100 µl/well of 1N H_2SO_4 and the absorbance was measured at wavelength 450 nm using a Bio-Rad 550 Microplate reader.

Statistical analysis

A T-test was used to assess statistical significance between the levels of either serum IgG or sIgA antibody obtained from mice immunized with OVA in solution and in microemulsion. P values < 0.05 were considered significant.

Results and discussion

In the present study, the mice were primed subcutaneously with OVA encapsulated in freeze-dried nanoparticles prepared using 6% (w/w) of ethyl cyanoacrylate monomer followed by oral booster of OVA formulated in either microemulsion or saline solution. The reasons behind this were based on both the finding of Chattaraj *et al.*, (1999) and the result of previous study (Pitaksuteepong, 2002). Chattaraj and co-workers (Chattaraj *et al.*, 1999) found that the magnitude of immune response was influent by routes of administration and the immune response to influenza viral vaccine given by subcutaneous priming followed by oral booster was shown to be high. In addition, in the previous study, the poly(ethyl cyanoacrylate) nanoparticles containing OVA were shown to initiate a higher immune response following subcutaneous administration, compared to OVA solution (Pitaksuteepong, 2002).

OVA-specific serum IgG antibody

Four weeks following subcutaneous priming with nanoparticles containing OVA, the IgG levels were low (Figure 2). As expected, at 3 weeks following oral boosting, the IgG levels were greatly elevated, which may contribute to the rapid and specific response of the memory B cells produced in the priming (Vitetta *et al.*, 1991). IgG responses to OVA formulated in the microemulsion was shown to be substantial higher than that to OVA solution. This may be due to the microemulsion has potential to enhance the absorption of the co-formulated as suggested by several findings (Ritschel, 1991; Charman *et al.*, 1992; Constantinides *et al.*, 1994; Constantinides, 1995).

At week 11, the IgG levels elicited by OVA formulated in both the microemulsion and saline solution, however, were decreased. The IgG responses induced by OVA in the microemulsion remained higher than those induced by OVA solution but they were not significantly different ($p = 0.11$). This may be due to great variation in the reduction of IgG level among the mice. A decrease in IgG responses at week 11 suggested that the immune response was not

maintained for a long period. This could be due to the phase inversion in the presence of large amount of gastrointestinal fluid, resulting in the burst release of the encapsulated antigen (Constantinides and Yiv, 1995).

Secretory IgA (sIgA) antibody

The similar results were observed in the levels of sIgA responses (Figure 3). The sIgA in faecal samples of mice immunized with both OVA solution and OVA formulated in microemulsion was increased substantially at 3 weeks following oral booster and was declined at 4 weeks later. Again, the IgA levels obtained from mice immunized with OVA formulated in microemulsion were higher than those obtained from mice immunized with OVA solution especially at week 7 ($p < 0.05$).

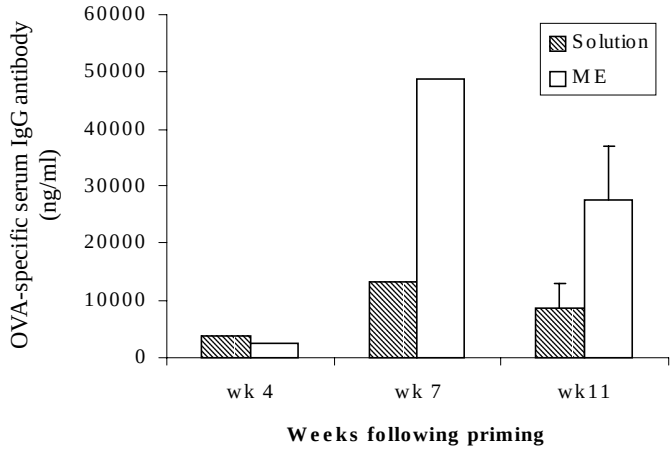

Figure 2. OVA-specific serum IgG antibody from Balb/c mice immunized with 0.5 mg OVA dissolved in saline solution (Solution) and medium-chain glycerides-based microemulsions (ME) at week 4, 7 and 11 (At week 4 and 7, values are measurement obtained from pooled serum while at week 11, values represent mean± SEM, n=8).

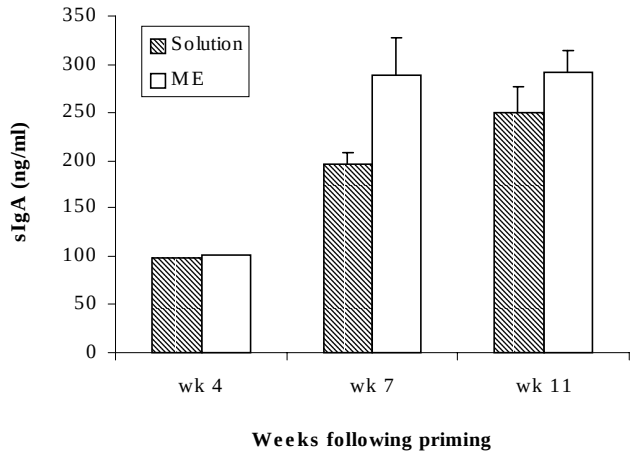

Figure 3. Secretory IgA in faecal extracts collected from Balb/c mice immunized with 0.5 mg OVA dissolved in saline solution (Solution) and medium-chain glycerides-based microemulsions (ME) on week 4, 7 and 11 (At week 4, values are measurement obtained from pooled faecal extracts while at week 7 and 11, values represent mean±SEM, n=8).

Conclusions

As discussed, microemulsions can enhance the intestinal absorption of labile and poorly soluble drug by altering mucosal membrane fluidity, resulting in the mucosal permeability changes. In addition medium-chain glycerides, naturally derived substances, were shown to enhance intestinal absorption. Hence, these properties of medium-chain glycerides-based microemulsions may enhance the absorption of antigen leading to a high immune response. Therefore, the investigation on the potential of a medium-chain glycerides-based w/o microemulsion for provoking immune response following oral administration using Balb/c mouse as a model was performed.

For cost-effectiveness purposes, the immunization via subcutaneous followed by oral booster was carried out because the priming could be successfully done using a single dose. Oral boosters using OVA in the microemulsion yielded higher IgG and sIgA responses than those using OVA dissolved in saline solution, indicating the potential of the medium-chain glycerides-based microemulsion for using as an antigen delivery system. However, the responses were not successfully maintained for long period. Thus it may be necessary to give repeated immunization. Further future work may need to incorporate some polymer into the microemulsion in order to prolong the release of the antigen and maintain the immune protection for longer period.

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