CHAPTER V

In vivo evaluation of Al(OH)$_3$ and chitosan conjugated PLGA microparticles as nasal Japanese encephalitis vaccine carrier

Introduction

Japanese encephalitis (JE) virus is a flavivirus caused a serious CNS disease in human, approximately 10,000-15,000 deaths each year in Asia, especially eastern and southeastern Asia. The vector of this disease is mosquitoes (Misra and Kalita, 2010; Solomon, Ni and Beasley, 2003). After a mosquito bite, viral replication occurs locally and migrates to regional lymph nodes. Virions disseminate to secondary sites, where further replication contributes to a viremia. Invasion of the CNS probably occurs from the blood by antipodal transport of virions through vascular endothelial cells. Infection in the CNS spreads by viral dissemination through the extracellular space or by direct intercellular spread. The principal clinical manifestation of illness is encephalitis. Encephalitis manifests with altered sensorium, seizures and focal neurological deficit. Illness usually begins with abrupt onset of high fever, change in mental status, gastrointestinal symptoms, headache follows gradually by disturbances in speech or gait or other motor dysfunction. Irritability, vomiting and diarrhea or an acute convulsion may be the earliest signs of illness in an infant. Seizure occurs more than 75% in pediatric. Conversely, headache and meningism is more common in adults (Misra and Kalita, 2010; Halstead and Tsai, 2004; Yang et al., 2004; Solomon, Ni and Beasley, 2003). Three JE virus control strategies have been considered based on transmission cycle of JE virus including vector control, swine immunization and immunization of humans. Anyhow, the human immunization remains the most reliable control for human JE at present (Halstead and Tsai, 2004). JE vaccine produced from mouse brain extracted (Flamand et al., 1995), requires three doses of subcutaneously administration and the booster are required depending on the risk of JE virus contacted at 6 years of age and once again at 10 years of age. In some area, boosters are given annually until age 10 (Halstead and Tsai, 2004). In order to increase the patient compliance and to reduce the side effect of subcutaneous JE vaccine caused by mouse brain protein, mucosal JE vaccine such as oral and nasal vaccine would be the great alternative options. According to in vivo study of nasal

vaccine, there are many factors affecting the immune response elicited by nasal administration of vaccine and vaccine entrapped particles, even if by the same vaccine. Among them are; dose of vaccine, volume of vaccine, frequency of administration, size of delivery particles, internal structure of delivery particles, surface hydrophobicity, surface charge and adjuvant effect (Okamoto et al., 2008; Jaganathan and Vyas, 2006; Hori, Onishi, and Machida, 2005; Yasuda et al., 2003; Husmann et al., 2002; Gutierro et al., 2002; Khang et al., 1999; Desai et al., 1997; Esperaza and Kissel, 1992). The ideal dose and frequency of each kind of vaccine is dissimilar, depending on immunological properties of vaccine, route of administration and type of adjuvant (Yasuda et al., 2003; Isako et al., 2000 and Sjölander, 1992). Therefore, ideal dose and frequency of each vaccine have to be evaluated whenever the system is changed. In contrast with the volume, large volume could elicit a great deal of titer via nasal route compared to small volume when given along with particle as the large volume could transport the fluid vaccine to the distal region which is the locality of Waldeyer's ring. Waldeyer's ring represented the collection of oropharyngeal tissues and nasal associated lymphoid tissues (Fujimura et al., 2004; Eyles, Williamson and Alpar, 1999).

Size is an important parameter to control the uptake of particles via nasal tissue (Desai et al., 1997). Nowadays, nanoparticles are widely employed as nasal vaccine carrier. However, huge nanoparticles with particle size of up to 1µm were reported to stimulate higher immune response when compared to the other diameters of nano-sized range particles such as 200, 500 and 800nm (Kanchan and Panda, 2007; Katare, Muthukumaran and Panda, 2005; Gutierro et al., 2002). Even though many research groups have investigated the effect of micron size particles, the results were enormously various and depended on many factors such as route of administration, type of vaccine and polymer used, for instance. Therefore, the effect of microparticle size on enhancing intranasal immune response is greatly of interest and needed to be greatly explored, especially for each specific type of vaccine along with the polymer of interest. In addition, surface hydrophobicity and surface charge are also the key factors for tissue adhesion which affected the stimulation of mucosal immune response. The hydrophobic positively charged particles prefer to adhere on mucosal surface followed by the great taken up into the underneath tissue (Fischer et al., 2006;

Janathan and Vyas, 2006; Hori, Onishi and Machida, 2005). As a result, the preparation of hydrophobic positively charged particles for nasal delivery has been an alternate. Internal structure of particles is not an imperative aspect on particle uptake, however it is incredibly influence to vaccine integrity after released (Panyam et al., 2003; Husmann et al., 2002). Thus, an experiment on interaction between vaccine integrity and the internal structure of particle supposed to be investigated as well. Adjuvant means "to help" as adjuvant could augment the immune response of antigen. Each adjuvant has its own advantages so it could be selected according to the use and route of administration in order to facilitate the achievement of each specific type of antigen (Matheis, Zott and Schwaing, 2002). In this study, PLGA, chitosan (CS) and aluminium hydroxide (Al(OH)$_3$) were selected to prepare the delivery vehicles as nasal JE vaccine carrier. Thus, the objective of this experiment was to study the effect of variable parameters which were dose, size and surface charge of free and encapsulated JE vaccine in PLGA, CS conjugated PLGA (1C) and Al(OH)$_3$ conjugated PLGA (1A) particles on enhancing the intranasal immune response of Balb/c mice. The large volume of vaccine was administered (Eyles, Williamson and Alpar, 1999), and the frequency was submitted to the available subcutaneously JE in the market which, in this study, could be adjusted according to the changes of the route. The excellent formulations of this study were selected for further experiment in particulate stability and vaccine integrity.

Materials and Methods

Materials

Poly (D,L-lactic co-glycolic acid) (PLGA) with lactide : glycolide 50 : 50 and BCA kit were purchased from Sigma-Aldrich (Saint Louise MO, USA). Polyvinyl alcohol, PVA, (MW. 67,000) was obtained from Fluka chemical (Switzerland). CS molecular weight of 37kDA with 94% degree of deacetylation was procured from Seafresh® (Bangkok, Thailand). Al(OH)$_3$ is a gift from Bureau of Veterinary Biologic (Bangkok, Thailand). JE vaccine was kindly provided by Government Pharmaceutical Organization (Bangkok, Thailand). All reagents used in SDS-PAGE were obtained from Sigma-Aldrich (Saint Louise MO, USA). The other chemicals were of analytical grade and used as received.

Experimental animals

Male Balb/c mice, 4-6 weeks old were obtained from the National Laboratory Animal Center, Mahidol University. Ten groups of five mice were housed with free access of food and water throughout the experiment.

Japanese encephalitis virus in vaccine

Purification and concentration of inactivated JE virus

Purification and concentration of JE virus were performed by Nanosep® device (Pall Life Sciences, USA). JE vaccine kindly provided from Government Pharmaceutical Organization, composed of inactivated JE viruses suspended in Media 199 solution in which the media was removed before using in this experiment by Nanosep® with membrane molecular weight cut off at 100K. Obtained purified JE was then concentrated by the same device and the amount of JE was determined by BCA assay using BCA kit and observed under UV-VIS spectrophotometer (Jasco V-530, Schimidzu, Japan) at 560nm.

Morphology and integrity of inactivated JE virus

The morphology of virus was examined by transmission electron microscope (TEM, JEM-1230, JEOL, Japan). The integrities were investigated by SDS-PAGE analysis. The conformation was determined by circular dichroism (CD).

Preparation of and characterization of particles

PLGA particles were prepared by double emulsion solvent evaporation technique using JE vaccine as entrapped material to obtain 1,5 and 15μm particles and subsequently conjugated 1μm particles with $Al(OH)_3$ and CS as previously described (Tunsirikongkon and Ritthidej., in press). In brief, an appropriate amount of purified JE vaccine was added into 8 ml of 5%PLGA in dichloromethane (DCM) with ratio of 1:10, 1:2.5 and 1:10 for 1,5 and 15μm particles, respectively and then sonicated by 3-mm diameter standard probe sonicator at output control of 20 for 10 seconds to form

primary w/o emulsion. Then, aqueous PVA of 4%, 4% and 2% were added into the primary w/o emulsion with the ratio of primary w/o : aqueous PVA at 1:2, 1:4 and 1:2 in order to obtain secondary emulsion for 1, 5 and 15μm particles, respectively. The input force to form the secondary emulsion was either by bath sonicator (for 15μm) or probe sonicator(for 1 and 5μm), correspondingly. The double emulsions were diluted in 100 ml of 1% w/v PVA and the solvent was eliminated by stirring up to 3 hours at 500 rpm. The resulting particles were washed and collected by centrifugation at 10,000 g for 5 minutes. The CS conjugated formulation was prepared by either adding 0.2% CS into the concave of centrifuged particles or blending 0.2% CS with 1%PVA at the final process of dilution. The volume of 0.2% CS added to the concave of centrifuged particles was excess in order to obtain the complete conjugation. The particulate formulation containing CS were then vortexed for 15 minutes and shaken for 2 hours subsequently. After shaking, the mixture was left overnight and the excess of un-conjugated CS was removed by centrifugation at 10,000g for 5 minutes. $Al(OH)_3$ conjugated formulation was obtained by adding 0.75% $Al(OH)_3$ into the concave of the centrifuged particles following the regulation of US code of federal regulations (610.15(a)) that the amount of aluminium is limited to $\leq$ 0.85mg for a single human dose of vaccine. As a result, the amount of aluminium in the vaccine given to Balb/c mice was calculated to not more than the limited amount. The physical properties of particles were investigated. The preliminary study of particulate stability was also evaluated by examine the characteristics of selected particles. Size and size distribution were evaluated by Laser diffractometer (Mastersizer 2000, Malvern, UK). Morphological evaluation was determined by scanning electron microscopy (SEM, JEOL, JSM-5410LV, Jeol, Japan). Surface charge analysis was established by photon correlation spectrophotometer (Zetasizer nanoseries, Nano-ZS, Malvern, UK).

Immunization

Dose- response relationship

Five groups of Balb/c mice (five mice per group) were either subcutaneously or intranasally administered with various doses of purified JE at day 0 and 7. The

booster dose was obtained at day 28 and the volumes of administration were at 100μl and 50μl for subcutaneous and intranasal administration, respectively as following protocol:

Group 1D: Subcutaneous immunization of 10μg dose in 100μl volume

Group 2D: Intranasal immunization of 10μg dose in 50μl volume

Group 3D: Intranasal immunization of 40μg dose in 50μl volume

Group 4D: Intranasal immunization of 80μg dose in 50μl volume

Group 5D: Intranasal immunization of 50μl PBS

Size-response relationship

Three groups of Balb/c mice (five mice per group) were intranasally immunized with 1, 5 and 15μm of JE encapsulated PLGA particles (JEP) at day 0, 7 and 28 with appropriate dose as following protocol:

Group 1S: Intranasal inoculation of 1μm JEP, with appropriate dose

Group 2S: Intranasal inoculation of 5μm JEP, with appropriate dose

Group 3S: Intranasal inoculation of 15μm JEP, with appropriate dose

Dose of vaccine was accorded from experiment of dose-response relationship.

Surface-response relationship

Two groups of Balb/c mice (five mice per group) were intranasally received Al(OH)$_3$ conjugated JEP and CS conjugated JEP at day 0 and 7 (No booster doses were required), with appropriate particle size and dose as following protocol:

Group1SF: Intranasal vaccination of Al(OH)$_3$ conjugated JEP, with appropriate particle size and dose

Group1SF: Intranasal vaccination of CS conjugated JEP, with appropriate particle size and dose

Dose and particle size were quoted from experimental of dose-response relationship and size-response relationship, respectively. After commencing the immunization, bloods were collected from mice at week 0, 2, 4, 6 and 8 and centrifuged to attain the serum. Serums were kept at $-20^{\circ}C$ until the assay. The antibody subclass IgG and IgA were determined by ELISA.

Antibody assessment : Enzyme linked-immunosorbent assay (ELISA)

The 96 well plates were coated with 100 μl per well of coating buffer, pH 9.6 containing JE antigen. The plates were thoroughly washed three times with phosphate buffer saline plus Tween 20 (PBST) pH 7.4 and allowed to dry before use. The 100 μl of blocking solution, 3% gelatin in PBST, was added to each well of the coated plate and incubated for 1 hour at $37^{0}C$. The plates were thoroughly washed three times with PBST and allowed to dry. The serum samples were added and internally two fold diluted with diluent (1% gelatin in PBST). The sample plates were incubated at $37^{\circ}C$ for 1 hour and then the plates were washed with PBST for three times. The 100 μl of horseradish peroxidase-goat anti-rabbit IgG and IgA, diluted 1 in 4,000 with diluent, was added to each well and incubated for 1 hour at $37^{0}C$. The plates were washed again with PBST for three times. After that, the 100 μl of the substrate, OPD, at a concentration of 1 tablet per 12 ml of citrate buffer plus 12 μl of 30% hydrogen peroxide, was added to each well. The plates were covered in dark and incubated for 30 minutes at room temperature for color development. The 50μl of 4N sulfuric acid was added to each well to stop the reaction. The plates were gently shaken and the optical density (OD) was read at wavelength 492 nm using a microplate reader (VICTOR[3], Perkin Elmer, USA). The end point was expressed as the last dilution which gave an optical density more than average baseline plus 0.05. The serum samples from each group of animals were studied to determine the titer range and the mean immune response at each time point.

Statistical analysis

Statistical method used is ANOVA. Normal distribution and homogeneity of variance of each experimental group were first examined before applying to one-way ANOVA program in the experiment of animal study.

Results and discussion

Characterization of JE virus

The morphology revealed by TEM showed that JE virus was a spherical virus at the approximate size of 50nm as shown in Fig 5.1. Normally, JE virus is in a spherical form possessing a lipid envelope with surrounds a densely staining core which corresponded to the image in this study. Staining by Uranyl acetate, the lipid envelop of virus can be seen, particularly the lipid envelope of purified JE virus as it was clearly stained compared to crude virus. SDS-PAGE is normally used for analysis of protein components by estimating the molecular weight. The molecular weight predicted of JE , are: 65-68 kDa for NS3, 53-54 kDa for envelop protein (E), 40-42 kDa for NS1, 18-22 kDa for intracellular glycoprotein precursor to M (prM), 10-14 kDa for capsid protein (C) and 8 kDa for unglycosylated membrane protein (M) (Misra and Kalita, 2010; Halstead and Tsai, 2004; Mutoh et al., 2004; Konishi et al., 2003; Wu et al., 2003; Kolaskar and Kale, 1999). In this study, crude JE presented in lane B as a smear band was a result of the contaminated proteins from mouse brain suspension as illustrated in Fig 5.2. Lane C and Lane D represented the different batches of purified JE vaccine appearing at about 68, 53, 42, 22 and 14 kDa which represented NS3, E, NS1, prM and C, respectively. The E or envelop protein could be considered as a main epitope of JE consisting of nearly 500 amino acids with two potential glycosylated sites which was important for the entry of the virus into the host cell and was the major target of humoral immune response (Misra and Kalita, 2010; Tajima et al., 2010; Ramakrishna et al., 1999; Ashok and Rangarajan, 1999; Konishi et al., 1997; Lin et al., 1996; Yeolekar and banerjee, 1996; Hasegawa et al., 1995). It was obvious that the immunological structure of JE used to elicit the

antibody response was remained in both crude and purified JE which could be concluded that the process of purification did not destroy the core immunological structures. The conformation of total protein of JE solution revealed by circular dichroism in Fig 5.3 was clearly in α-helix rich structure as the molar ellipticities were found at around 226 and 208, respectively.
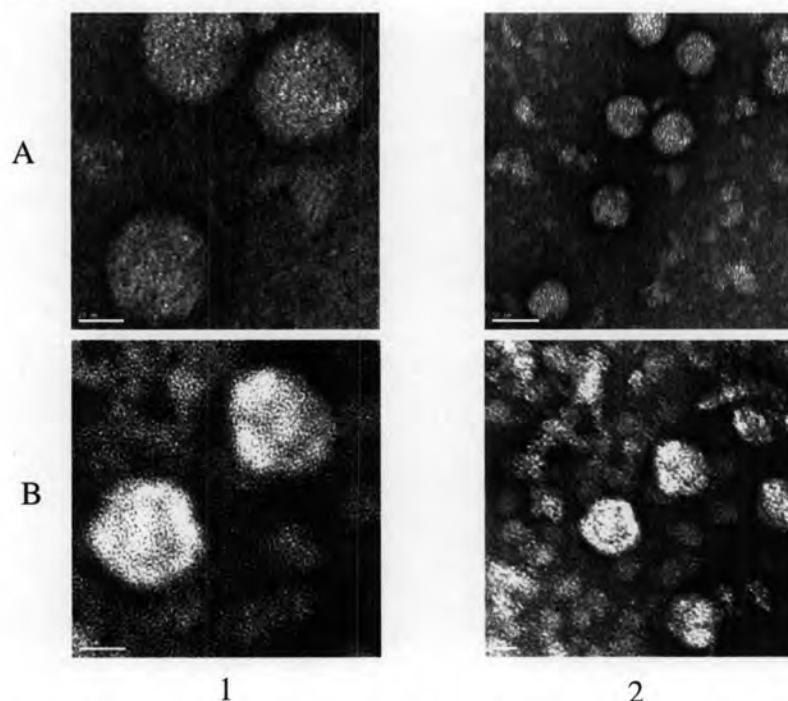

Figure 5.1 Morphology of purified JE vaccine, (A1-A2) and crude JE vaccine, (B1-B2) by TEM
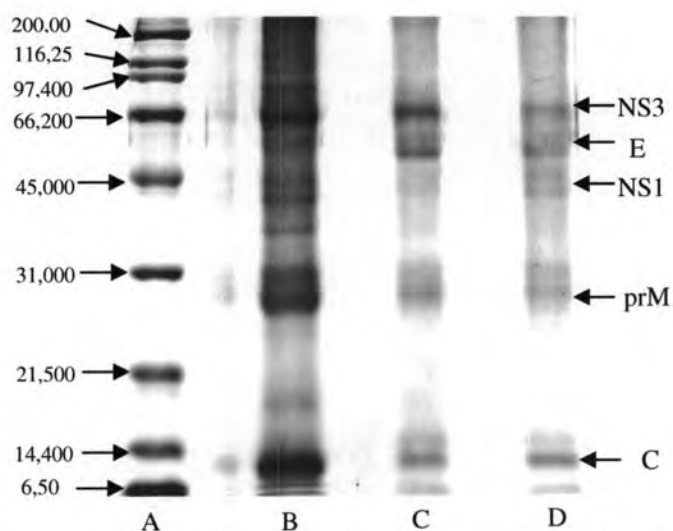

Figure 5.2 Protein profile by 12% gel of SDS-PAGE of JE vaccine, Lane A: Molecular weight marker, Lane B: crude JE vaccine, Lane C and D: purified JE vaccine

Obviously that after purification and concentration of JE vaccine by Nanosep® device, the conformation of total protein was transformed gradually from α-helix rich to β-sheet rich since the intensity of ellipticity at around 208 was progressively disappeared and conversely stronger to the ellipticity of 226. When the concentration of JE was increased by purification, the intensity of ellipticity at 226 was even higher and tended to shift to the lower wavelength to β-sheet rich structure at 220 as previously reported (Tunsirikongkon and Ritthidej, in press). The shift from α-helix rich to β-sheet rich structure of total JE protein was a result of an entanglement between molecules of protein which probably caused by an increasing of JE concentration after the purification process (Tanaka et al., 2001; Kelly and Price, 1996). The E protein epitope along with other surface proteins of JE could still be observed by SDS-PAGE after purification and concentration process. Thus, the antigenic epitope of E protein could still effectively stimulate JE immune response.

Characterization of particles

In our recent study, we reported the characteristics of new delivery particles of CS conjugated PLGA and Al(OH)$_3$ conjugated PLGA particles (Tunsirikongkon and Ritthidej, in press). The physical properties of selected formulations were shown in Table 5.1.
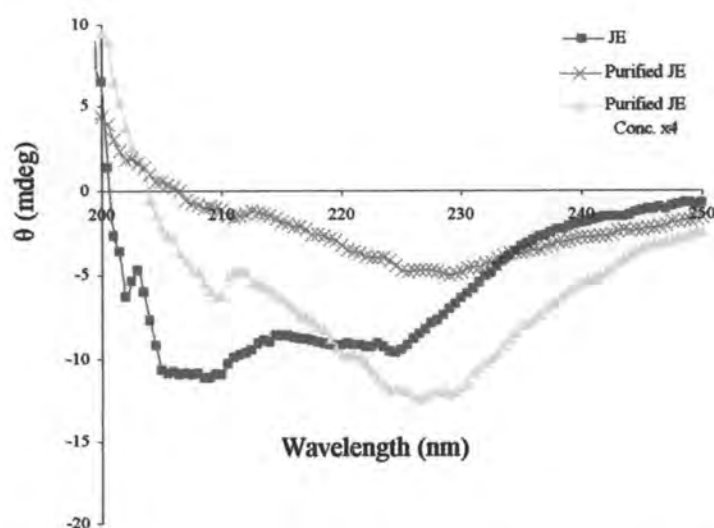

Figure 5.3 Protein characteristics by CD of JE vaccine, purified JE vaccine and purified JE vaccine at 4 times concentration.

It was also observed that surface charges of conjugated particles, 1C and 1A, were positive while surface charges of un-conjugated particles were mildly negative. SEM pictures of particles expressed the smooth and spherical shape except formulations of Al(OH)$_3$ and CS that Al(OH)$_3$ formulation demonstrated the rough surface of particles as a result of the coated Al(OH)$_3$ on particles surface while the surface of CS particles was covered by the layer of transparent gel of CS as shown in Fig 5.4, A1, B1 and C1, respectively (pictures of 5 and 15μm are not shown).

Stability of selected particles and vaccine integrity

It was observed that by approximately two months (8 weeks), the size of 1μm particulate formulation was slight increased from 0.92 to 2.13μm while the uniformity value was increased considerably from 0.27 to 11.3 as illustrated in Table 5.1. The size of conjugated particles were, however, noticeably shifted from 1.22 to 104.48 μm for CS and from 1.62 to 13.26 μm for Al(OH)$_3$, respectively. The uniformity value of both conjugated formulations were, surprisingly, decreased from 7.72 to 1.17 for CS and from 1.59 to 0.71 for Al(OH)$_3$, correspondingly.

Table 5.1 Physical properties of selected PLGA formulations

Formulations	Zeta potential (mv±SD)	Median size (μm), uniformity	
		Initial	Week 8
PLGA 1μm	-5.38±0.13	0.92, 0.27	2.13, 11.3
PLGA 5μm	-7.42±0.31	5.26, 1.57	Not determine
PLGA 15μm	-6.90±0.86	16.76, 2.03	Not determine
1C	+22.17±2.67	1.22, 7.72	102.48, 1.17
1A	+15.36±1.27	1.62, 1.59	13.26, 0.71

The morphology of formulations revealed by SEM in Fig 5.4 illustrated that all conjugated and un-conjugated particles were in the spherical shape in week 0. However, conjugated particles of both CS and Al(OH)$_3$ tended to stick together by an

adhesion of each single particle while 1μm particles were relatively liberated from each other. Within two months, 1μm particles had a tendency to group more as a cluster which corresponded to the results of size and uniformity that size and uniformity were both increased by two months. However, the shape of all particles of 1μm formulation remained in the spherical form which indicated that most particles were rarely degraded. According to CS formulation, particles of this formulation were grouped together since week 0 and seemed to be coalesced and degraded more and more according to time as the shape of particles was merely in the spherical form and become irregular shape. Moreover, the number of particles observed was less gradually according to time as well. This result corresponded to the results of size and uniformity shown in Table 5.1 since particle sizes were greatly larger by the particulate coalescence and these coalesced particles were grouped together into irregular shape which resulted in a small value of uniformity. At week 8, only small amount of spherical particles of CS formulation was observed. The CS formulation tended to be the most degradable formulation compared to 1μm and $Al(OH)_3$ formulation. According to $Al(OH)_3$ formulation, it was presented that particles appeared to be adhered more into a cluster by the network of $Al(OH)_3$ according to time until week 8.

At week 8, $Al(OH)_3$ particles grouped collectively to a stumpy cluster which corresponded to the result of size and uniformity that the measured sizes was more immense and the uniformity value was, nevertheless, more minute. However, particles of $Al(OH)_3$ at week 8 were remained in spherical shape specified that the degradation rate of $Al(OH)_3$ formulation was slower than that of CS formulation. The final in vitro degradation products of PLGA were oligomer of lactic acid, oligomer of glycolic acid, lactic acid and glycolic acid. The degradation started immediately after an immersion into a medium (Avgoustokis et al., 2002). Because of this reason, the pH of medium decreased by time according to the degradation product of PLGA. The particles prepared by PLGA 50:50 started degrading obviously from approximate day 35 determine by the sharply dropping of pH. The degradation occurred when the MW of polymer decreased to a very low value until the molecular chains were small enough to freely diffuse out into surrounding media even though the total mass lost was stabilized (Yang et al., 2008; Li and Chang, 2005)
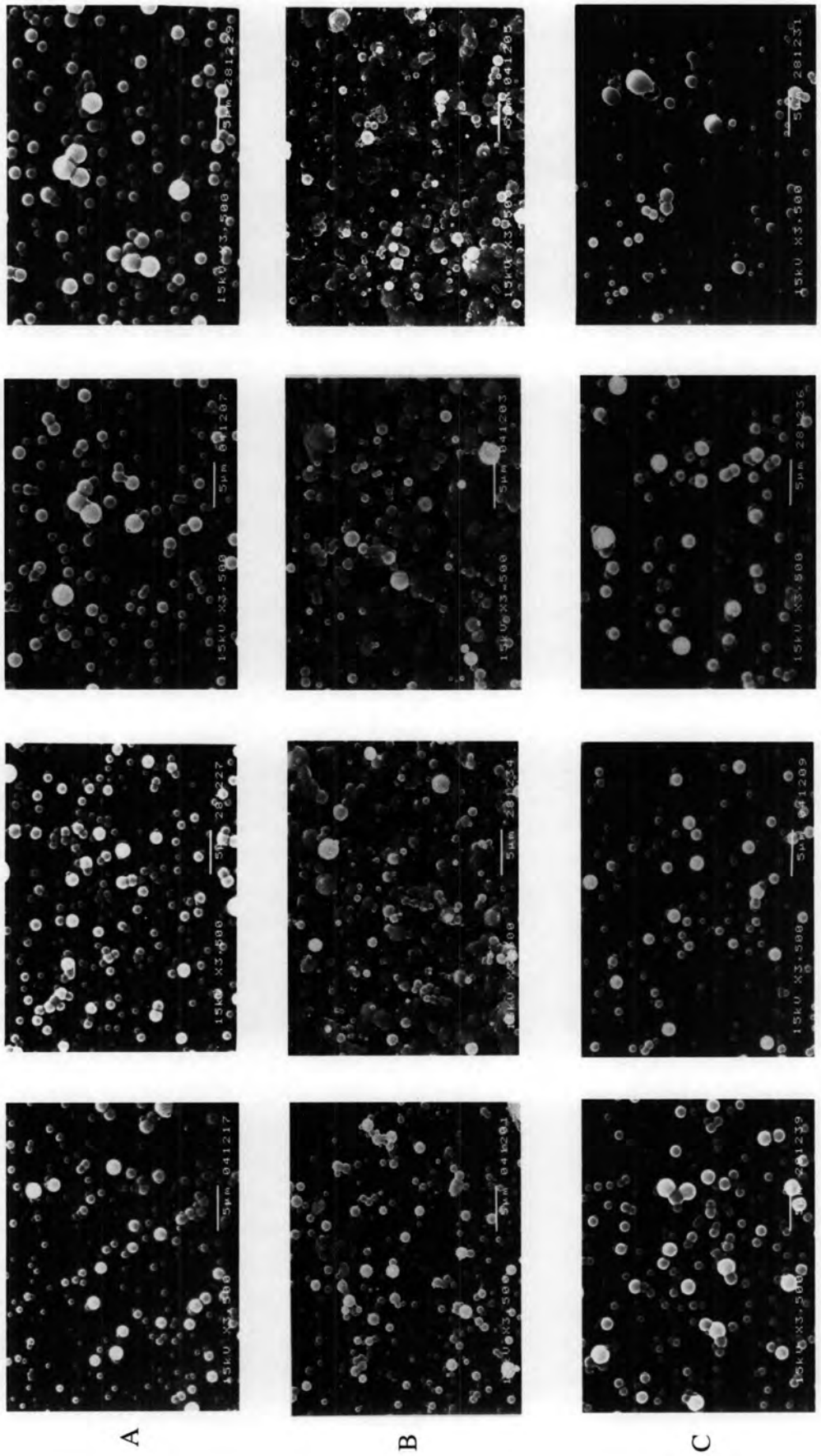
Figure 5.4 Morphology of 1μm PLGA particles at week 0, 1, 2 and 8 (A1-A4), Al(OH)$_3$ conjugated PLGA particles at week 0, 1, 2 and 8 (B1-B4), and CS conjugated PLGA particles at week 0, 1, 2 and 8 (C1-C4) observed by SEM (x3,500)

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This would result in either the lost of spherical shape or the merge of particles as corresponded to both conjugated and un-conjugated formulations in this study. The higher degradation rate of conjugated particles might attribute to an increasing of particulate hydrophilicity by conjugated CS which apparently increased the wettability of particles to water phase. Degradation of the CS conjugated PLGA depended on both the surface erosion of CS phase and the bulk hydrolysis of PLGA phase (Mi et al., 2002; Avgoustokis et al., 2002; Siegel et al., 2002). This was probably the reason for the degradation rate of CS formulation which was the most rapid among all. Rapid particulate degradation in vivo would also be an advantageous because it would result in rapid polymer removal from body which prevented the polymer accumulation in the case of multiple particulate application (Avgoustokis et al., 2002). According to Al(OH)$_3$ formulation, particles were hold as a cluster by a fine gel-like network of alum in which this network formation ability was evident as early as 30 minutes and considerably increased when incubated with particles for prolonged periods of time (Kanchan et al., 2009; Katare and Panda, 2006). Moreover, the degradation product of PLGA consequence in the pH change of the system which resulted in the different pattern of Al(OH)$_3$ gel formation. Additionally, the charge of released JE might probably play an important role in formation of aggregated particles which corresponded to the result in this study. Regarding to the protein conformation of surface JE proteins, the conformation of total proteins of JE released from Al(OH)$_3$ formulation was barely investigated by CD while the total proteins conformation of JE released from CS formulation could relatively be observed but could not be identified the exact structure as shown in Fig 5.5. However, the results of immunological response indicated that antigen interconnected to the network of adjuvant such as Al(OH)$_3$ and CS could remain stimulate the immune response. Though, the protein characteristics of JE released from 1, 5 and 15µm were in the α-helix rich structure as the molar ellipticity were found at around 226 and 208, respectively, as shown in Fig 5.6, which corresponded to the conformation of JE after contacting with organic solvent of dichloromethane (DCM) during the preparation process of particles. The epitope proteins of JE with DCM were also evaluated by SDS–PAGE from previous study (Ritthidej and Tunsirikongkon, in press) and

expressed that all epitope proteins of JE were very well retained after contacting with DCM.
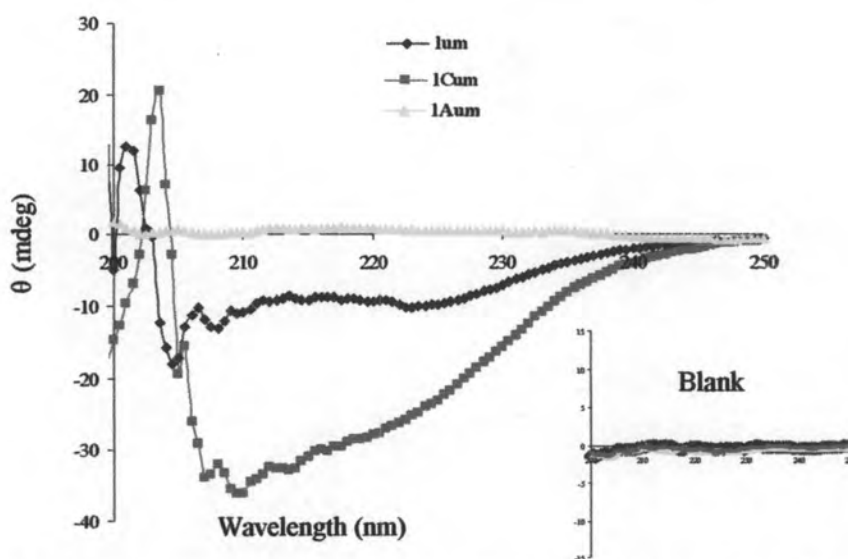

Figure 5.5 Protein characteristics by CD of JE recovered from 1μm, CS conjugated 1μm (1C) and Al(OH)₃ conjugated 1μm (1A) PLGA particles
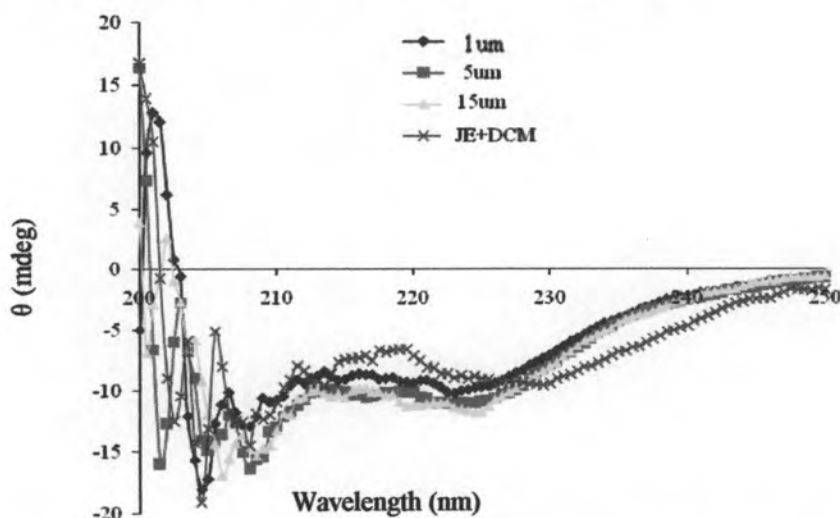

Figure 5.6 Protein characteristics by CD of JE recovered from 1μm, 5μm, 15μm PLGA particles and JE with DCM

Immune response

Dose-response relationship

According to the antibody titer, IgG of mice immunized with 10μg subcutaneously JE vaccine was considerably high, with about 24735.69, at week 2

after the two times of immunization. The titer reached the peak (50335.69) at week 4. The titer was relatively stabilized until the end of experiment as shown in Fig 5.7A.

For intranasal immunization, mice immunized with 10, 40, and 80µg JE elicited lower IgG level with statistically significant ($p<0.05$) for all doses compared to mice immunized with 10µg JE subcutaneously at all of blood sampling times. The IgG of mice immunized intranasally was gradually increased and tended to be continuously increased after the booster dose, except for the 80µg dose of intranasal JE that the immunological titer tended to decrease at week 6. The antibodies IgA were detected in all mice with fairly low titer for both subcutaneous and nasal route as illustrate in Fig 5.7B. However, the mean IgA titer of nasal route was relatively more than PBS group and also slightly more than subcutaneous route. However, the levels of IgA at all points of blood sampling were exceedingly low down when compared to IgG according to all doses of vaccine. PBS was also inoculated as negative control for both IgG and IgA and both obtained the low immunological response.

The titers of all groups tended to be stable until week 6 except IgG of mice immunized intranasally with 80µg dose and IgA of mice immunized intranasally with 10µg dose that the titer of these groups were considerably declined from 2623.92 to 925.12 and from 102.06 to 67.04, respectively. However, the differences among IgG titers of three JE doses were statistically insignificant ($p>0.05$) while 10µg dose elicited IgA was statistically significant different from the other doses of vaccine ($p<0.05$). The decline of IgG might be a consequence of a too high dose of vaccine (80µg) that the antibody which was just generated was mopped up by the new administered antigen. Moreover, mucosal tolerance mechanism could play an important role for the reduction of antibody response in that this mechanism was signified to protect the body from extended mucosal exposure to the high amount of antigen resulting in a systemic T cells hyporesponsiveness consequence in a lower systemic immune response (Mestecky, Moldoveanu and Elson., 2005). The involved T cells in this mechanism could be both Th1 and Th2. The systemic hyporesponsiveness of T cells derived mediator was noticeably regulated by the regulatory cytokines named, TGFβ, IL-10 and IL-4, which produced mainly by mucosal T-cells and epithelial cells at mucosal site for the purpose of switching and terminal differentiation of B cells into IgA-producing cells (McGhee et al., 1992).

Thus, the suppression of systemic immune response might occur by the process of mucosal tolerance. Additionally, it can be concluded that the relationship between the antibody response and dose of vaccine would only be demonstrated over the specific range of antigen amount of each specific kind of vaccine (Bock et al., 1990) corresponding to the result of this study. For JE, the dose of antigen intended for nasal application was not able to reach 80µg. However, the immunization interval had to be co-considered in addition to the dose of vaccine (Greenbaum et al., 2004).

The ability of nasal associated lymphoid tissue (NALT) to induce an immune response at a distal mucosal site and in blood circulation may lie in part within the specialized ligands expressed by its high-walled endothelial venules (HEV) (Csencsits et al., 1999). The ligands or addressins were interacted with specific homing receptors expressed by B and T lymphocytes and allow the trafficking of these lymphocytes from effective site to distal lymph tissue. All NALT HEV express PNAd, either alone or in conjunction with MAdCAM-1 or VCAM-1 which could interact with α4β7 surface receptor and recirculated via endothelial blood vessel to the distal target site (Mackay, 1993). The extravasation of lymphocyte through endothelial venules was influenced by microenvironmental control in that the expression of chemoattractant/activating factors released from endothelial cells of each target site was capable of initiating secondary adhesive mechanisms and vessel transmigration, and finally the regulation and maintenance of homing receptor expression patterns on lymphocytes (Picker, 1994). B lymphocyte could be differentiated and activated by Th cell at either local site which produced IgA or in blood circulation that normally generated IgG in order to switch to the antibody producing cell. According to IgA, the sIgA (B-cells) could be activated by isotype specific CD4$^+$ Th cell which expressed specific receptor in order to enhance the differentiation of B cells from sIgA to IgA antibody producing cell which produced IgA antibody (McGhee et al., 1992). There might be an opportunity for sIgA to encounter the CD4$^+$ Th cell along the HEV trafficking pathway in blood circulation and switch to IgA antibody producing cell which probably be both specific and non-specific IgA producing cells. Even though the level of IgA antibody observed in blood circulation in this study was considerably lower than IgG, it could presumably be replied that there were the migrated sIgA in blood circulation which could switch

to IgA producing cell and secrete the specific IgA antibody titer. The specific IgA antibody was detected by ELISA in which the ELISA plates were coated by purified JE. From the results of IgA in blood circulation, it could be concluded that IgA antibody in external secretion of nasal tissue was as well be obtained. Regarding to the infectious site of JE, the study of Raengsakulrach et al. (1999) has shown that exposure of monkeys to JE virus via intranasal route developed the encephalitis in Rhesus monkeys, thus, both mucosal and systemic IgA were important and presumably able to be the first defense barrier for JE infection.

The selected dose of JE for further experiment of intranasal immunization in this study was 10μg as it was sufficient and comparable to evoke the intranasal IgG to 40μg dose and the dose of 80μg was too high and conversely mopped up the contributed antibody response. Even though the IgA response of 10μg dose was less than 40μg and 80 μg, the noticeably higher level of IgG supposed to be more considered for JE protection as JE virus was infected by mosquitoes bite via the skin and the viral replication occurred locally at the site of administration and disseminated to the secondary sites which would end up at the CNS via blood circulation. To protect this infection, the systemic immune response was more required in order to obliterate the virus in blood circulation (Okuno et al., 1987). Therefore, the IgG titer (systemic immune response) is more necessary rather than IgA antibody titer. Thus, 10μg dose was selected. The dose-response relationship was also studied by Jaganathan et al. (2006) and found that the dose-dependent response was considerably observed by immunizing guinea pigs with various doses of TT (0.3, 0.5, 0.7, 0.9 and 1.1 Lf/dose) and 0.5 Lf/dose triggered the highest levels of antibodies while immunization with >0.5 Lf/dose showed decreasing antibody production which could refer that the too high dose was most likely inappropriate to prompt the immune response.

The highly difference in immunological level between nasal and subcutaneous immunization might be a consequence of many factors. The nasal mucociliary clearance could be recognized as mainly barrier that the vaccine solution could not defeat in order to penetrate through the mucosal membrane. Additionally, the enzymatic degradation in nasal cavity was also be another effective barrier (Davis, 2001).
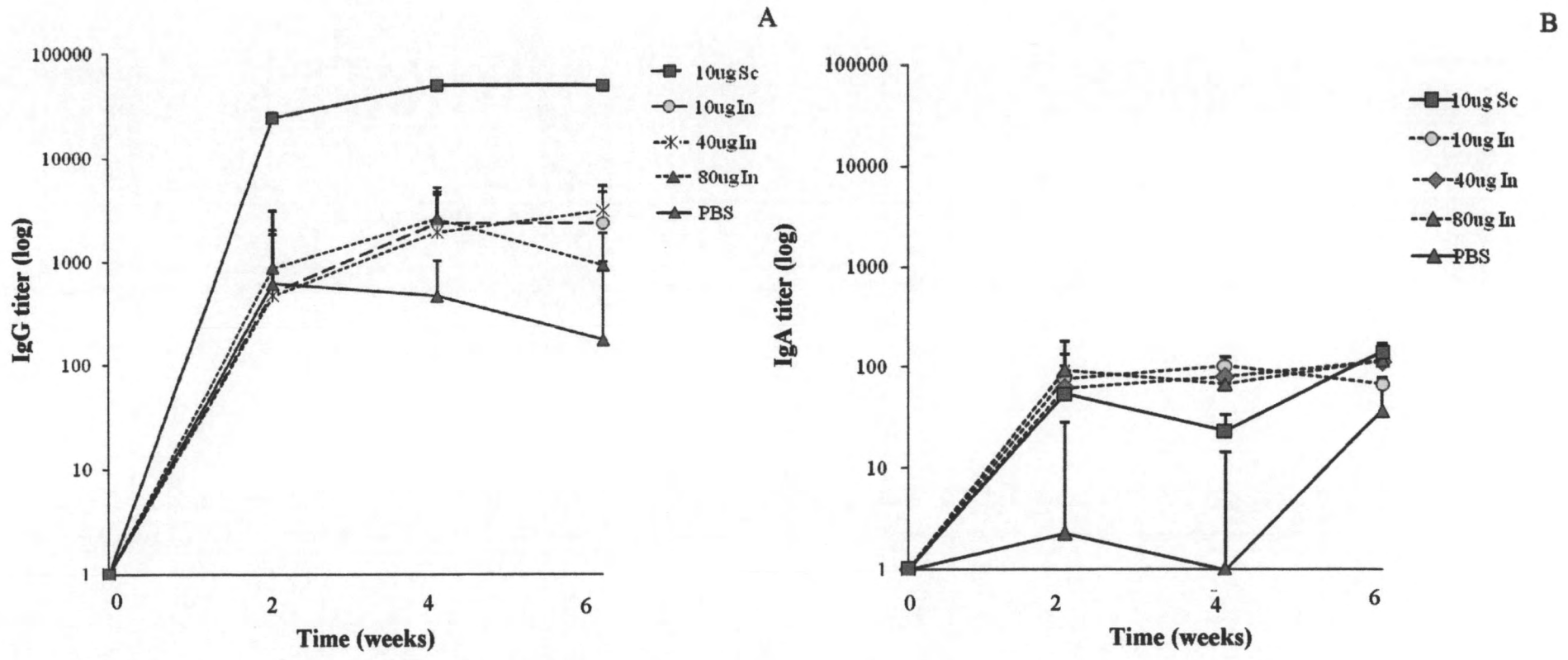
Figure 5.7 IgG (A) and IgA (B) response of mice immunized with different doses of purified JE vaccine via intranasal and subcutaneous route using PBS as control group

Thus, the mucosal delivery particles had to be developed in order to avoid the mucociliary clearance and enzymatic degradation. In order to evoke an equal level of intranasal immune response with subcutaneous vaccination by using the same dose of vaccine (10µg), mucosal delivery system such as the delivery particles would be a further compulsion.

Size-response relationship

Mice were immunized with different sizes of PLGA particles entrapped JE vaccine which were 1, 5 and 15 um, respectively for an intention to enhance the mucosal immune response. Dose of vaccine used for each time of immunization was equally at 10ug for all sizes of particles regarding to the result of dose-response relationship from the above result. The titers of all sizes of nasal formulations were compared to subcutaneous administration with the same dose by mean of their antibody titer.

The antibody titer, IgG of all sizes were observed at week 2 after two times of immunization, with the titer of 2380.36, 1213.52 and 577.3 for 1, 5 and 15µm particles, respectively and increased gradually until week 4 as shown in Fig 5.8A. At week 4, booster dose was inoculated to all groups. Even though the booster dose was inoculated to all groups of experimental animal, the antibody response to 1 and 15µm particles were declined at week 6 while the response of 5 µm particles seemed to be quite stable. However, at week 8, the titer of mice immunized with 1µm particles were increased considerably to 16187.89 whereas the response of 15µm was slightly raised to 4039.67 and the titer of 5 µm particles was remained stabilized. From the results, it could be concluded that the delivery particles of PLGA with particles size of 1µm tended to enhance the statistically higher IgG antibody titer of vaccinated Balb/c mice (p<0.05) compared to 5µm, 15µm and purified vaccine. However, the booster dose was still necessary to stimulate and maintain the antibody titer of particulate vaccine of all sizes. The level of antibody response could be ranked as 1 µm >5 µm >15 µm ≈ purified JE vaccine solution, respectively, according to the same dose. However, the antibody level of 15µm particles was relatively comparable to the purified JE vaccine solution since large size required more time to release antigen

resulting in less accumulated antigen in the system which consequence in the dropped down of antibody titer till less than purified vaccine. Though, the titer level of 15µm was gradually increased at week 8 by the more release of antigen in the system after the booster dose. Nevertheless, the particulate vaccine could considerably elicit the higher antibody titer compared to non-particulate vaccine which corresponded to the finding of Gutierro et al. (2002).

The main advantages of particulate delivery were the protection of antigen from enzymatic degradation and the avoidance of elimination by mucociliary clearance compared to antigen itself. Additionally, particulate carriers could also enhance the high level of immunological response by progressively release of the antigen. In case of micron sized particles, there was a constant liberate of antigen from particles for a considerable period of time and micron size as well was preferred to be taken up by specific type of cells. Furthermore, plasma membrane of cells had more capacity to process and present antigen from micron size particles and this might be the dominant mechanism for antigen presentation following immunization with microparticles (Kanchan and Panda, 2007). The particles shape and size could also be manipulated to maximize interactions at the cellular level.

Phagocytotic cells such as macrophages and M-cells were able to ingest micron-sized particles with diameters between 1 to 5µm, therefore, targeting these cells in the intestinal or respiratory tract would allowed the use of large particles. In contrast, only particles that were in the nano-sized range could be endocytosed by epithelial cells via the intracellular or paracellular pathway but less efficient of antigen processing and presentation by phogocytotic cells (Chadwick, Kriegel and Amiji, 2010; Bramwell and Perrie, 2006; Katare, Muthukumaran and Panda, 2005). This would be the reason why 1 and 5µm size particles could augment more response as they were both preferred to interact with M-cells which could be considered as immunological component of mucosal immune response and endocytosed by mucosal epithelial cells in the case of 1µm particles in order to process for antibody production. For that reason, 1µm particles enhanced further titer compared to other sizes of particles such as 5µm and 15µm particles.
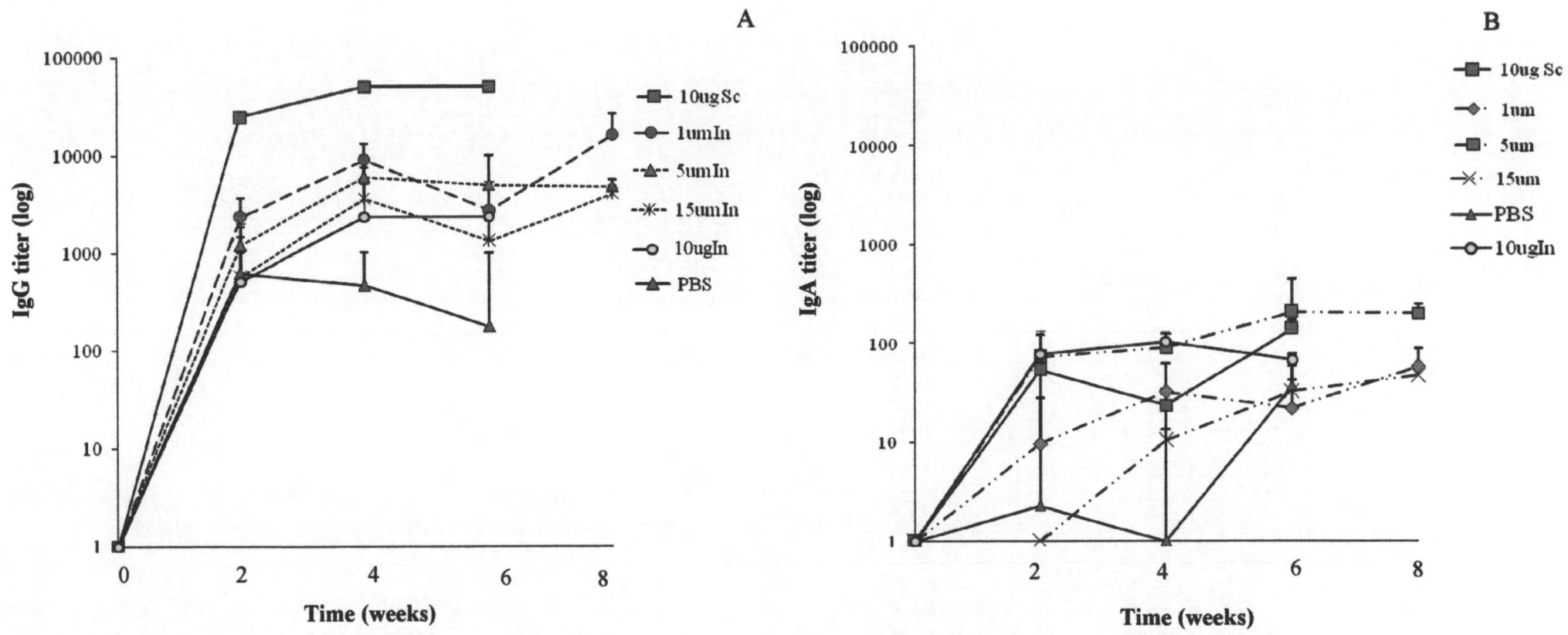
Figure 5.8 IgG (A) and IgA (B) response of mice immunized with purified JE vaccine encapsulated in different sizes of PLGA carriers, via intranasal and subcutaneous route using PBS as control group

Moreover, by the fact that 1and 5μm formulations obtained more number of small particles within the formulations in nano-sized range compared to 15μm, the immune response generated via epithelial cells uptaking to blood circulation was additionally recognized resulting in a high systemic antibody of IgG. According to 15μm particles, the available surface area of the large particles for the attachment to antigen presenting cell was lower when compared to small particles which might be the result of the low generated antibody titers (Kanchan and Panda, 2007; Katare, Muthukumaran and Panda, 2005; Davis, 2001). One more reason to explain the differences in the immunological response of particles with various diameters would be the fact that different size of spheres had a quantitatively different distribution in the lymphoid tissues (Gutierro et al., 2002). This hypothesis was as well corresponded to the results of antibody and morphology which illustrated by SEM in this study. Considering to the endocytosis process of particles with different sizes, uptake can be divided by using mechanism of clathrin-mediated endocytosis and clathrin independent uptake mechanism in which both mechanisms depended mainly on size of particles (Xiang et al., 2006). For the particles size of larger than 500nm such as 1 μm and 5μm in this study, the key process of uptake was clathrin independent mechanism which occurred mainly via macropinocytosis and phagocytosis by specialized cells known as sentinels of the immune system, probably be the M-cells. After uptake, macropinosomes expressed as large irregular vesicles of 0.5-5μm diameter and generated when membrane protusion fused back with plasma membrane. As a result, the small size of 1 and 5μm could be taken up by macropinisomes into the vesicles while larger size of 15μm could not. This mechanism could enhance the antibody titer of small size of particles apart from the normal process of nasal cell taken up.

Regarding to IgA titer, the results revealed that particulate vaccine stimulated only small level of antibody titer in which all groups were not more than 204.78 as illustrated in Fig 5.8B. Additionally, the levels of antibody titer of both particulate and non-particulate vaccine were not more than 250 that could be considered as very small amount. However, particles of 1 and 5μm in size seemed to stimulate more level of antibody compared to 15μm as 15μm formulation could not at all stimulate the antibody response before week 2. Nevertheless, the titer of mice immunized with

particulate and non-particulate vaccine was different in immunological pattern as particulate vaccine acquired more time to raise the titer compared to non-particulate vaccine. Moreover, elicited titer from particulate vaccine tended to be gradually increased and persisted for a longer period of time, especially 5µm particles.

The result suggested that IgA in serum could serve as a marker for IgA in mucosal secretion which corresponded to the finding from Jaganathan and Vyas (2006) that specific IgA could be detected in the sera of mice immunized intranasally with either PLGA particles alone or CS coated PLGA particles of hepatitis B, but not in sera of mice immunized through subcutaneous route. As a result, local titer of IgA could be implied from IgA in sera. Moreover, the study from Borges et al. (2008) indicated that IgA immune response of particulate adjuvant could as well be generated the high level of antibody titer even by the immunization of negatively surfaced charge particles that was able to enhance both mucosal and systemic immune response which corresponded to the results in this study. Thus, particles of negative surface charge could still enhance the IgA immune response and the generated titers were increased slowly but persisted for a long period of time. After two times of immunization, the antibody titers of all groups increased gradually and started declining after week 4 of all sizes for IgG and only 1µm formulation for IgA. Then, IgG and IgA of all groups started increasing again after the second weeks of booster dose held at week 4. Though, the IgA titer for 15 µm formulation could be obviously observed with the higher level only after the booster dose. Hence, the administration of booster dose was required in order to maintain the antibody level of particulate vaccine. This could be due to the un-modified PLGA microspheres which might be fastly cleared from the nasal cavity at the initial stage soon after nasal administration. Thus, the microspheres supposed to be modified to render mucoadhesive property in order to enhance the clearance half-life.

Surface-response relationship

After two times of immunization on day 0 and 7 by formulations of mucoadhesive PLGA particles of CS (1C) and Al(OH)$_3$ (1A), the antibody titers, IgG of both 1C and 1A groups observed at week 2 were relatively high, with about

4537.45 and 4104.23, respectively, even higher than the titer of 1μm particles which was only at 2380.36 as shown in Fig 5.9A. For the vaccinated mice by both 1C and 1A formulations, the booster dose was not critically necessary as the titer was sufficiently high and obviously persisted until week 6 to 8 and remained lofty, with about 19683.42 and 10313.45 for CS and Al(OH)$_3$ formulations at week 8, respectively. The CS formulations seemed to raise slightly higher antibody response than the Al(OH)$_3$ formulation at all blood sampling times. However, the antibody titers of both 1C and 1A groups at week 8 showed the statistically insignificant different (p>0.05) while at week 6, both 1C and 1A represented the higher immunological titer compared to 1μm particles as well as purified JE vaccine with statistically significant different (p<0.05). For IgA titer, nevertheless, the titers of both conjugated groups were not initially high compared to IgG. The response increased gradually and reached the peak at week 6 for Al(OH)$_3$ formulation (341.47) and at week 8 for CS formulation, with about 328.16, respectively as shown in Fig 5.9B. Though, the levels of titer of both conjugated formulations were much higher and remained stabilized compared to un-conjugated formulation without any need of booster dose. The presence of positive surface charge increased muco-adhesion by interacting with a negative charge of cell membrane or mucus layer in order to help the facilitation or internalization of antigen and delivery particles (Thomas et al., 2009) and to reduce the clearance rate of particles (Jaganathan and Vyes, 2006).

This study supports the hypothesis that positively surfaced charge particles could reduce the clearance rate from the nasal cavity, thereby increasing the contact time of the delivery carriers with the nasal mucosa (Jaganathan and Vyas, 2006; Kaur, Rauthan and Vrati, 2004). Particle surface chemistry and size also play an important role in deciding the cellular localization of polymer particles (Kanchan and Panda, 2007). Thus, negatively surfaced charge of suitable size PLGA particles in this study was modified by positively charged substances of CS and Al(OH)$_3$ for the purpose to enhancing the immune response by increasing both mucosal adhesion as well as cellular adhesion and localization. CS could lend particles the mucoadhesive characteristics resulting in reduced elimination rate compared to those of un-conjugated particles (Jaganathan and Vyes, 2006) and obtained more time to be taken up by mucosal tissue consequence in the higher level of antibody titer.
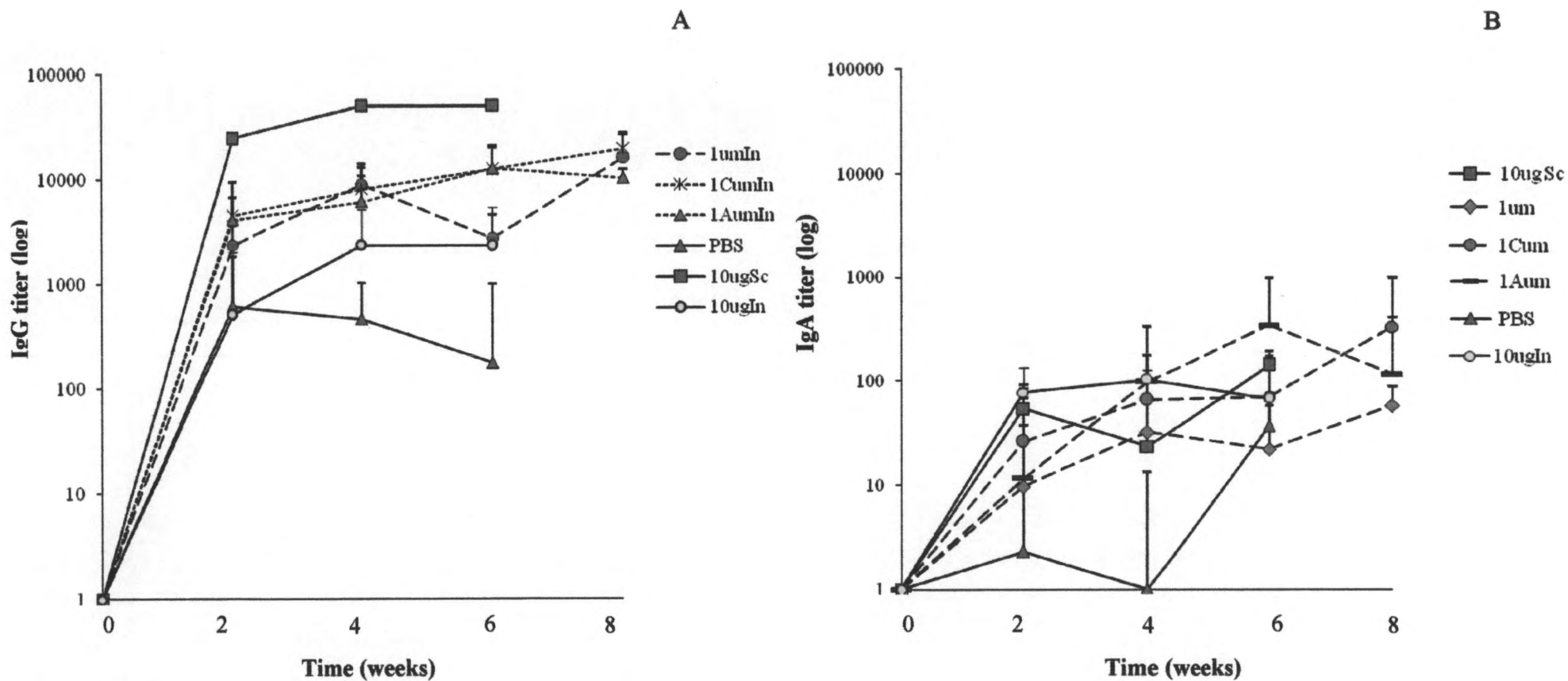
Figure 5.9 IgG (A) and IgA (B) response of mice immunized with purified JE vaccine encapsulated in 1μm PLGA carrier and conjugated with CS (1C) and Al(OH)₃ (1A), via intranasal and subcutaneous route using PBS as control group

Furthermore, un-charged and positively charged particles consisting of hydrophobic polymer such as PLGA provided an affinity to mucosal tissue such as intestinal mucosa as well as to adsorptive enterocytes, whereas negatively charged particles showed the lower affinity to any types of cells and tissues (Jung et al., 2000) in which this finding could possibly be implied to the other side of mucosal tissue such as nasal mucosa. CS was also very efficient in transporting vaccines to M-cells such as M-cells in nasal tissues and Peyer's patches, and opening the tight junction, resulting in an enhancement of both local and systemic immune responses and the production of toxin neutralizing antibodies (Van der Lubben, 2003). As a result, CS was the hydrophilic polymer with positively charge which could enhance the affinity of particles to mucosal tissue and gained the relatively high level of antibody titer (Mundargi et al., 2008; Jaganathan and Vyes, 2006; Van der Lubben, 2003).

According to $Al(OH)_3$, alum formed a network of gel-like structure when used along with particles and held particles in form of small clumps. Because of this reason, particles were held together by alum while getting attached to the surface of both tissues and antigen presenting cells. This was the reason of improving the immune response after the immunization with admixture of alum and particles (Kanchan, Katare and Panda., 2009; Katare, Muthukumaran and Panda, 2005; Morefield et al., 2005). Besides, it had been reported that amount of antigen released from particles in vitro in presence of alum were lower than that from particles alone since the polymeric formulations were associated with burst release of antigen, presence of alum helped in reducing the burst release by adsorbing the antigen and presented the antigen to antigen presenting cells (Kanchan, Katare and Panda, 2009). This suggested that antigen released from particles was adsorbed on alum and presented in a better way to improve the antibody response. Apart from this, alum also facilitated the tissue inflammation leading to macrophage activation, thus enhanced the antibody response when used along with particle for immunization (Hansen et al., 2007; Brewer, 2006; Katare, Muthukumaran and Panda, 2005; Raghuvanshi et al., 2002). Accordingly, alum obtained many characteristics to improve and enhance the immunological response.

The IgG response of Balb/c mice immunized via intranasal route with no booster dose by conjugated formulations gave an identical pattern to mice immunized

via subcutaneous route with booster dose. The pattern of antibody titer was started by rising and then stabilized again. From the results, two doses of conjugated vaccine were enough to evoke and maintain the nasal immune response comparable to subcutaneous pattern. In summary, an appropriate dose of 10µg JE entrapped in the conjugated particles of 1µm size with no requirement of booster dose could enhance an efficient nasal antibody response of JE. This study was undertaken to evaluate the efficacy of nasal immunization of mice with different dose, carrier sizes and modified surface charge of carriers. The obtained results were in the figures of antibody titer IgG which represented systemic immune response and IgA which represented mucosal immune response. As the model vaccine in this study was JE which infected and developed in blood circulation, the efficacy of systemic immune response was greatly in our interest. Though, as the study from Raengsakulrach et al. (1999) illustrated that JE could also be developed and infected via nasal route, the local IgA has raised its own value. According to the study from Ramakrishna et al. (1999), oral immunization of JE vaccine generated the high antibody titer in blood of IgG at geometric mean of 8854 using the evaluation technique of ELISA with the level of neutralizing titer at 30. With this level of titer, Ramakrishna found that the percentage of protection after the challenge by JE virus was relatively high, with 76.75% while the subcutaneous immunization gave 100% of protection. The percentage of neutralization which could represent the protection efficiency was corresponded as well to the raise of antibody level, IgG in many findings (Appaiahgari et al., 2006; Wu et al., 2004; Konishi et al., 2003; Nam et al., 1999; and Raengsakulrach et al., 1999) in which these findings could reply to the results in this study. According to the results in this study, the highest antibody level IgG among three immunized doses without any carrier was 3110.68 while after encapsulating in different sizes of carriers, the titer was significantly raised to more than 8854 for formulation of 1µm at week 8 with 16187.89 in level. Furthermore, after conjugation of 1µm carrier with CS, the IgG level was raised to more than 8854 since week 6 with the titer of 12607.88 and reach 19683.42 at week 8. After conjugation with $Al(OH)_3$, the titer at week 6 was 12820.12 and only slightly dropped to 10313.45 at week 8. This could be concluded that intranasal immunization with 1µm particles encapsulated JE, CS conjugated 1µm particles encapsulated JE and $Al(OH)_3$ conjugated 1µm particles

encapsulated JE obtained an extremely high possibility to protect the challenge and JE infection of more than 76.75% protection since week8, week6 and week6, respectively.

Conclusions

Efficiency of different doses of JE vaccine administered via intranasal route using PLGA and conjugated PLGA particles of different size and surface charge was evaluated in experimental animal of this study. The results indicated that there was a dose response relationship of JE vaccine administered intranasally within the specific range of dose from 10μg to 40μg. However, the highest dose should less than 80μg in this study as a new generated antibody was eliminated by the new administered antigen of too high dose. The size response relationship of delivery particles was obviously observed after nasal administration of 1, 5, and 15μm particles and found that particles of smaller than 5μm could induce typically a high antibody titer of systemic immune response due to their susceptibility to be distributed in mucosal associated lymphoid tissue. However, the levels of elicited titer were not comparable to commercially subcutaneous vaccine within the same frequency of administration owing to the negatively surfaced charge of particles. The combination of positive surface charge of mucoadhesive substances such as CS and $Al(OH)_3$ with delivery particles seemed to be favorable to assist the adhesion of particles on tissue surface which could help the particles to overcome mucosal barrier. The antibody response of mice immunized with both CS and $Al(OH)_3$ conjugated particles were considerably higher than un-conjugated particles without any booster doses as a result of their positive surface charge and the pattern of immunological response which was relatively identical to mice immunized via subcutaneous route with booster dose. Though, both conjugated particles significantly enhanced the systemic immune response after nasal delivery, there was less observed for the serum antibodies, IgA. However, the systemic immune response of IgG was more essential for this particular vaccine of JE to eliminate the virus which infected via skin and circulated in blood circulation. This study have shown promise of new particulate technology for typical JE vaccine as nasal carriers and the further work of in dept analysis in stability study is also required and now in an ongoing progress.