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นายอนันต์ วัชรดำรงกุล

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IMMMUNE RESPONSE OF RABBITS AGAINST LIPOSOME CONJUGATE OF *PASTEURELLA MULTOCIDA* POTASSIUM THIOCYANATE EXTRACT

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การศึกษาคุณสมบัติทางเคมีกายภาพ คุณสมบัติในการเป็นอิมมูโนเจนในการกระตุ้นการตอบสนองของภูมิคุ้มกันและการทดสอบการฆ่าเชื้อในหลอดทดลองของอิมมูนซีรัมของไลโปโซมที่บรรจุสารสกัดโปตัสเซียมไทโอไซยาเนตของเชื้อพาสเตอเรลลามัลโตซิดา 8A และ 2T35 โดยขนาดของไลโปโซมที่ได้มีขนาดในช่วง 23.34 และ 30.12 ไมโครเมตรและเปอร์เซ็นต์ของโปรตีนที่อยู่ภายในไลโปโซมอยู่ในช่วง 59.69-63.69 การวัดแอนติบอดีไตเตอร์ของอิมมูนซีรัมด้วยวิธีอีไลซาโดยทำปฏิกิริยากับสารสกัดของเชื้อชนิดเดียวกันพบว่าซีรัมจากกระต่ายที่ฉีดไลโปโซมมีค่าแอนติบอดีไตเตอร์สูงสุด เมื่อเทียบกับซีรัมของกระต่ายที่ฉีดอลัมและสารสกัดโปตัสเซียมไทโอไซยาเนต

การศึกษาความสัมพันธ์ของการตอบสนองของภูมิคุ้มกันและผลการฆ่าเชื้อของอิมมูนซีรัมที่จำเพาะกับไลโปโซม อลัม และสารสกัดโปตัสเซียมไทโอไซยาเนตของเชื้อพาสเตอเรลลามัลโตซิดา 8A และ 2T35 โดยทดสอบแอนติบอดีไตเตอร์ด้วยวิธีอีไลซาต่อสารสกัดโปตัสเซียมไทโอไซยาเนต และเชื้อพาสเตอเรลลามัลโตซิดาที่ทำให้ตายด้วยความร้อน พบว่าการตอบสนองต่อไลโปโซมสูงสุด ขณะที่การตอบสนองต่ออลัมและสารสกัดโปตัสเซียมไทโอไซยาเนตจะต่ำกว่า การฆ่าเชื้อของอิมมูนซีรัมจะได้ผลเช่นเดียวกันและพบว่ามีความสัมพันธ์ระหว่างแอนติบอดีไตเตอร์ต่อเซลล์แบคทีเรียและผลการฆ่าเชื้อของอิมมูนซีรั่มต่อเชื้อพาสเตอเรลลา 8A และ 2T35

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Liposome containing potassium thiocyanate extract of *Pasteurella multocida* 8A and 2T35 were studied on their physicochemical properties, immunogenicity to induce immune response, and the in vitro bactericidal effect of immune sera. The particle size of liposomes were in the range of 23.34 and 30.12 μm and the percent protein entrapment of liposomes were in the range of 59.69 to 63.69. The antibody titer of immune sera were determined by ELISA with homologous strain and the result indicated that liposome induced the highest antibody titer compared to that of alum and KSCN extract.

The correlation of the immune response and bactericidal activity of immune sera induced by liposome, alum, and potassium thiocyanate extract of *P. multocida* 8A and 2T35 was investigated. The antibody titer was determined by ELISA against potassium thiocyanate extract and heat killed *Pasteurella multocida.* The highest immune response was produced from liposome, while the lower response were produced by alum, and potassium thiocyanate extract. The similar result was obtained from the bactericidal activity. There was good correlation between antibody titer against whole cell and bactericidal activity of antiserum against *P. multocida* 8A and 2T35.

Department	Microbiology	Student's signature	
Field of study	Microbiology	Advisor's signature	
Academic year	2000	Co-advisor's signature	

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ABBREVIATIONS

BSA	Bovine serum albumin
°C	Degree(s) celsius
CFU	Colony foming unit
CMC	Sodium carboxymethyl cellulose
DCM	Dichloromethane
g	gram
i.n.	Intranasal
kDa	Kilodalton
Kg	Kilogram
KSCN	Potassium thiocyanate
LPS	Lipopolysaccharides
M	Molar
mg	Milligram
µl	Microliter
µm	Micrometers
µ	Micron
min	Minute
ml	Milliliter
MLV	Multiplevesicular liposome
N	Normal
nm	Nanometer
NSS	Normal saline solution
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffer solution
PBS-T	Phosphate buffer saline solution with tween-20
PC	Phosphatidylcholine
%	Percent
ppm	Part per million
s.c.	Subcutaneous
TBA	Tryptic soy blood agar

CHAPTER I

INTRODUCTION

Vaccination against infectious disease is an attractive alternative to chemotherapy. Although, conventional vaccines in the forms of live attenuated or killed organisms can induce protective immunity, unacceptable side effect such as mortality and systemic infection may still be induced(1). Advanced technology tend to be resulted in the development of subcellular or antigen vaccines, because these vaccines can elicit specific immune responses and can eliminate undesired pathogens. Unfortunately, these subcellular vaccines are usually weak immunogens in the absence of an immunological adjuvant(2).

Adjuvants are agents that can induce strong immunity to antigens. They appear to function by creating a depot at site of injection and activating macrophages (3). At present, there are only a few adjuvant used such as aluminium hydroxide (alum), saponin complex, and Freund's adjuvant. Only alum is licensed for safety to be used in humans, but it is not always efffective adjuvant. The others are toxic, inducing granulomas, acute and chronic inflammations, cytolysis and pyrogenicity (4,5). For these reasons, the new generations of adjuvants are desired and liposomes are investigated attractive adjuvant candidates.

Liposomes may be simply defined as phospholipid vesicles consisting of phospholipid bilayer enclosing an aqueous comparment. They were brought to the attention as models of cell membranes by Bangham A.D. in 1965 (6). Later, liposomes were investigated in a new role as immunological adjuvant when they were first demonstrated by Allison and Gregoriadis in 1974, using diphtheria toxoids(7) as an antigen, and had subsequently been confirmed and expanded in studies of many antigens(8-11).

Liposomes have several suitable properties for an immunological adjuvant(4). The first, since composed of natural lipids, they are biodegradable and nontoxic. The second, they readily disperse from site of injection to the draining lymph nodes, and do not cause granulomas or other local reaction. The third, they are sustained release system, function as an antigen depot. The last importance is their ability to induce cell mediated immunity(CMI). Although liposomes were considered to be good but there are also several problems in developing liposomal vaccines including the difficulty in

producing liposomes on a large scale, the expensive cost of phospholipid as raw materials, and the stability of liposomes during storage.To overcome these defects, various methods of liposome stabilization were tried. The two methods concluded effectively were adjusting cholesterol content of liposomal bilayer(12-17), and coating the surface of liposome vesicles with polymers(18-27).

Pasteurella multocida is a gram negative pathogenic bacteria which pathogenesis in animals frequently cause of great economic loss(28). The important diseases caused by *P. multocida* were fowl cholera in avian species(29,30) and hemorrhagic septicemia in cattle(31-33). To improve the safety of the fowl cholera vaccine, the immunogenicity of antigens or subcellular extract of *P. multocida* was investigated(34-43). The former studies showed that an antigen from *P. multocida* by KSCN extract was effective and could stimulate protective antibodies against homologous challenge in cattle, mice and rabbits(34-74).

The purposes of this study are

1.To determine the physical properties of liposomes containing protein extract from *P. multocida.*

2.To determine the immune response to liposomes containing protein extract from *P. multocida* in rabbits.

3.To determine the *in vitro* bactericidal capacity of immune sera.

CHAPTER II

REVIEW OF LITERATURE

Pasteurella multocida

Taxonomy

Rosenbanch and Merchant 1939 proposed the alternative name of *Pasteurella multocida* in Bergey's manual of Systemic Bacteriology(44). The genus Pasteurella can be divided among six species (Table 1) based on the beta hemolysin, growth on MacConkey agar , indole production, urease activity , gas from carbohydrates and acid production from lactose or mannitol. These species are *P. multocida, P. heamolytica, P. pneumotropica, P. ureae, P. aerogenes, and P. gallinarum.*

Morphology, growth characteristic and biochemical reaction

The usual appearance is that of small coccobacillus gram negative bacteria , 0.25 to 0.4 by 0.6 to 2.6 micron in size(45,46) . They were stained more distinctly at either pole, giving rise to the term bipolar. Bipolar staining is common especially in preparation made from blood and infected animal tissue. They are generally capsulated, but non-capsulated form has been reported. The majority of the species are non motile and spore forming. After repeated culture on agar, the bacteria trend to form longer rods and to become more pleomorphic, forming chains, filaments and rods of various sizes. When growing in carbohydrate media for prolonged periods, marked pleomorphicity is noted. The organisms usually possess a capsule. The capsules are mostly composed of hyaluronic acid . The bacteria is an aerobe and facultative anaerobe. Its optimum temperature for growth is at 37 °C, and the maximum growth is reached in 18 hours in fermentor or shaker condition. The pH growth range is from pH6-8.8 with an optimum pH of 7.2 – 7.4. The use of digested protein media or protease peptone stimulates the growth of the organism. Bacteria could be grown in beef infusion media but better growth is obtained when blood or serum is added to the media. Some strain fail to grow in media not containing blood or serum. Three principle colony types are seen on clear screen agar (4): (1) a smooth or (fluorescent), virulent for rabbits, growing diffusely in broth, whitish ,generally

Table 1 Differential characteristics of the genus *Pasteurella* (a)

Characteristic	*P.multocida*	*P.pneumotropica*	*P.haemolytica*	*P.ureae*	*P.aerogenes*	*P.gallinarum*
Hemolysis	-	-	+	-	-	-
Growth on MacConkey's agar	-	-	+	-	+	-
Indole production	+	+	+	-	-	-
Urease activity	-	+	-	+	+	-
Gas from carbohydrate	-	-	-	-	+	-
Acid production from:						
Lactose	-	d	d	-	-	-
Mannitol	b+	-	+	+	-	-

a Data from Carter (1981). For symbols see standard definitions.

b Strains from dogs and cats may be negative for mannitol

d Differs among strains

Table 2 Designation of serotypes of *P. multocida* by the Carter-Heddleston method

Capsular type	Somatic type	Serotype	Disease
A	1, 3, 4	A:1	Fowl cholera
		A:3	
		A:4	
	5, 6	A:5	Fowl cholera
		A:6	(Less common)
	7-10	A:7, A:8, A:9	
	12-15	A:10, A:12, A:13	
		A:14, A:15	
	16	A:16	Fowl cholera (turkeys)
B	2	B:2	Hemorrhagic septicemia
D	11	D:11	Atrophic rhinitis
E	2	E:2	Hemorrhagic septicemia

Table 3 Designation of serotypes of *P. multocida* by the Namioka-Carter method

Capsular type	Somatic type	Serotype	Disease
A	1, 3, 5, 7, 9	1:A	Pneumonia
		3:A	Pneumonia
		5:A	Fowl cholera
		7:A	Septicemia
		8:A, 9:A	Fowl cholera
B	6, 11	6:B	Hemorrhagic septicemia
		11:B	Wound infection
D	1, 2, 3, 4, 10	1:D, 2:D and 10:D	Pneumonia
		3:D	Pneumonia
		4:D	Pneumonia
E	6	6:E	Hemorrhagic septicemia

unstable, pathogenic and forming smooth ,moderately opaque iridescent colonies on serum agar ;(2) a rough or non-capsulated colonies (blue colonies), dewdrop-like and of a relatively low virulence. Giving a granular deposit in broth, and forming translucent bluish colonies ;(3) a mucoid form of intermediate virulence. The highly virulent smooth form contains a type-specific polysaccharide capsular antigen; the mucoid form is rich in hyaluronic acid, and may or may not possess a polysaccharide capsular antigen in addition. The rough form has neither a capsular nor a mucoid antigen . Strains giving rise to blue colonies are most frequently recovered from infection . While acute infection usually yield strains giving rise to fluorescent colonies . Many group A strains produced mucoid variants on initial isolation on enriched media.

All strains of *Pasteurella multocida* produce acid but not gas in glucose and sucrose; most strains ferment galactose, mannitol, mannose, sorbitol and xylose. Dextrose, dulcitol, lactose, maltose, and raffinose are occasionally acidified; inulin, inositol, rhamnose and salicin are usually not attacked. Strains from certain sources trend to possess particular biochemical characteristics (45). Strains of *Pasteurella multocida* are without action on litmus milk and gelatin. They produce indole, reduce nitrate and form a small quantity of H_2S as detected by lead acetate paper. The MR and VP reaction are both negative; the catalase and oxidase reaction are both positive ,though rather weakly so. Methylene bule is reduced . Citrate cannot be used as the sole source of carbon.

Serotyping(46)

The classification that gained widespread acceptance was based on serological properties of the organisms , capsular and cell wall compositions. At the present time , *Pasteurella multocida* have been identified as 5 capsular types designated A,B,D,E, and F and 16 somatic types. The popular method of designating serotypes was combined both capsular and somatic types (Table 2,3).

The former serotyping system is identified by Arabic numbers 1 through 16 representing the somatic type followed by a capital letter representing the capsular type. The important serotype which are the cause of fowl cholera are 5:A, 8:A and 9:A and of hemorrhagic septicemia are 6:B and 6:E.

Antigenic structure

The structure and cellular component of *Pasteurella multocida* may contribute to disease or stimulate host immune response. The major antigenic components are given below(48).

Capsules

The antigenic specificity of the capsular determines its serogroup (39). Capsular type A is composed of hyaluronic acid, which may act to mimic host antigenics because hyaluronic acid naturally present in host tissue. Intimate associated with its other polysaccharides, proteins and lipids. The hyaluronic acid does not exert antiphagocytic activity, but saline-extractable capsule material contained a factor capable of inhibiting the function of bovine polymorphonuclear leucocyte. The capsule of avian strains provided production from the action of complement. Removal of the hyaluronic acid capsule increased both the adhesiveness of the organism to animal cell surfaces and its susceptibility to phagocytosis. The production of capsular material was affected by subminimal inhibitory concentration of antibiotics. Capsule was considered virulence factor because no encapsulated variants of pathogenic strains were less virulent than encapsulated forms.

Lipopolysaccharides (LPS)

Examination of strains from a variety of animal sources confirmed that LPS from *P.multocida* was similar to semirough LPS of Enterobacteriaceae (49). The LPS contained lipid, 2-keto-3-deoxyoctanate (KDO), heptose, glucose and polysaccharide. The LPS of rabbit isolates contained either a non-serospecific antigen (R-LPS), a serospecific antigen (S-LPS) or both, from avian strains found to be R-LPS. The LPS responsible for antigenic specificity of somatic serotype, and when examined electrophoretically the molecular weight of LPS was low.

Toxins

The production of protein toxin by *P. multocida* has been recognized for sometimes although it is only in the last decade that some definition has been given to the nature of these products, following the discovery of toxigenic strain. Some strains,

particular those of capsular type D (49,50) produced a factor designated demonecrotic toxin (DNT). The toxin was antigenic ,heat labile ,protein in nature and purified DNT was a protein estimated molecular weight ranging from 112 – 160 kDa (51). Treatment of mildly trypsinized toxin with dithiothreitol and sodium dodecyl sulphate produced 3 polypeptide chains of molecular weight 23, 67, and 74 kDa from which the whole toxin could be reconstructed (52). Crude toxin was inactivated by formaldehyde, heat and proteolytic enzyme suggesting it was a protein (53). Commercial vaccines therefore incorporate formaldehyde treated whole cell of toxigenic *P.multocida* or formaldehyde- detoxified crude bacterial extract of toxigenic organism (54). Strains from other hosts also produced the toxins including poultry, calves, cats and dogs, rabbits and human respiratory tract (55). Dermonecrotic type-A strain could produce turbinate atrophy similar to that caused by dermonecrotic type-D strains.

Outer membrane protein

Outer membrane protein (OMP) from *P. multocida* was extracted and its ability to immunize animals against *P.multocida* infection and enhancing the virulence in infected animals through the anti-phagocytic mechanism were investigated.

Abdullahi et al.(56,57) studied the outer membrane proteins (OMP) profile of *Pasteurella multocida* type A and the related Taxon 13 strains isolated from bovine pneumonia to determine the heterogenicity of this group and to attempt to define a protective immunogen in a mouse septicemia model by investigation of the immunogenicity of the OMPs. They concluded that in the mouse model of pasteurellosis, the major OMPs were not protective antigens and therefore may be unlikely canidates for vaccines. However, their conclusions appear to contradict the findings of Lu et. al.(58) who showed that 31.5 kDa OMP (presumably outer protein) elicited protection against homologous challenge in a rabbit model, but in a mouse model, protection was afforded against heterologous challenge only if the challenge strain was shown by probing with a monoclonal antibody, to express the 37.5-kDa antigen. Protection was not provided against strain lacking this antigen. They showed that OMP can be a protective immunogen in the strains that possessed it, but they concluded that antigen may be more important as vaccine candidates. Because only

24 % of their strain of *Pasteurella multocida* expressed this antigen . Out of ten major polypeptides of OMPs in the extract of *Pasteurella multocida* serotype B:2, immunoblotting showed that the polypeptide with the OMP vaccine or a commercial HS vaccine developed the highest mean $\log_{10}$ ELISA titers 21 days post-vaccination. The results suggested that OMP was protective and could be used in vaccines against HS.

Antigenic extract from *Pasteurella multocida*

Potassium thiocyanate extract prepared from a virulent type A which contain protein , carbohydrate, lipopolysaccharide, DNA, and RNA was safe and protected rabbit against homologous challenge(59).

A vaccine was prepared from potassium thiocyanate extract of *Pasteurella multocida* (serotype 3,12,15:D)(60). Rabbits vaccinated intranasally or intraconjunctivally produced serum IgG and nasal mucosal IgA against *Pasteurella multocida*, whereas rabbits immunized by the intramuscular route(34) produced persisting serum IgG and transient nasal IgA antibodies. The hyperimmune serum directed to the KSCN extract of 3:A *Pasteurella multocida*(61) provided significant protection against homologous challenge in rabbits.

It was reported that intranasal immunization with both *Pasteurella multocida* toxin and potassium thiocyanate extract (62) induced an effective response against homologous *Pasteurella multocida* challenge. Coadministration of *cholera* toxin with potassium thiocyanate extract (63) enhanced protection immunity to *Pasteurella multocida* disease and infection in rabbits.

Plasmids

Avian strains contained plasmids and its characteristic together with a degree of complement resistant were correlated to virulence marker (64,65). Plasmids confering antibotic resistance have been isolated from fowl cholera and bovine pneumonia strains.

Protective antigens of *P. multocida*

Prevention of disease by using killed and live vaccine can induce protective immunity, but sometimes the results in moratlity, morbidity and systemic infection

are important problems. For these reasons, many researchers attempt to an improved vaccine efficacy by the determination of the immunogenic antigens or subcellular materials. There are numerous reports of studies in which vaccine have been prepared by extraction of antigens(66-72).

An antigen extracted from *P. multocida* type A by an aqueous solution of 0.5 M KSCN in 0.08 M sodium chloride was found to be immunogenic in chicken against homologous and heterologous challenge (34). Subcellular fraction from 2.5% sodium chloride solution contained capsular antigen (73) and complex substance with high molecular weight (40% protein and 15% carbohydrate) was immunogen. Truscott, 1988 , reported that all of turkey inoculated with this antigen survived from challenge (74).

Antigen extracted from a virulent isolate of *Pasteurella multocida* (serotype 3,12,15:D)with potassium thiocyanate was prepared as vaccine by Ringler et al .1985 (59). Pasteurella-free rabbits were vaccinated intranasally and intraconjuctivally twice with a 2-week interval and challenged intranasally with the homologous *P. multocida* 2 weeks after the second vaccination. The vaccinated rabbits produced serum IgG and nasal mucosal IgA against *P. multocida* . The vaccine protected the challenged rabbits from cilnical disease and death.

Jarvinen et al .1998(73) reported that subcutaneous (s.c.) immunization with either exotoxin or thiocynate extracts of *P. multocida* induced partial protection in rabbits. Since disease begins at mucosal sites. Induction of local immunity may be improtant in preventing systemic disease. Little is known concerning the efficacy of intranasal (i.n.) administration of these antigens in inducing protective mucosal immunity to *P. multocida* in rabbits. The effectiveness of vaccination with purified *P. multocida* toxin (PMT) and a potassium thiocyanate extract of *P. multocida* (CN) in combination was investigated (62). Rabbits received either one or both antigens by either s.c. or i.n. administration . Following vaccination, each group receive an i.n. challenge of *P. multocida* . Rabbits vaccinated with both antigen i.n. or s.c. had a 100% survival rate, few or no bacteria in the liver and lungs, high serum IgG and IgM antibody titers, and significant number of IgG antibody–secreting cells (ASC) in the spleen and tracheobronchial lymph node . Rabbits vaccinated i.n. has significant nasal and bronchoalveolar lavage IgA antibody levels . Rabbits vaccinated with one antigen, either PMT or CN , lower antibody titers, moderate to severe liver and lung

infections, and fewer ASC compared to rabbits receiving both antigens. Rabbits in the control groups had moderate to severe liver and lung infections. This study indicated that i.n. immunization with both PMT and CN induced an effective response against homologous *P. multocida* challenge.

Lu et al., 1991 (74) identified protein immunogens of OMP by radioimmunoprecipitation (RIP) and western blot analysis. They demonstrated that rabbits mounted major antibody response against 27 , 37.5 , 49.5 , 58.7 and 64.4 kDa OMP. They further demonstrated that vaccination with OMP protected rabbits against homologous challenge. More specifically, Mab against 37.5 kDa OMP protected both mice and rabbits against challenge. *P. multocida* lipopolysaccharide (LPS) has similar chemical and biological properties to the R – type LPS of the gram negative bacteria. Purified LPS is antigenic, however the level of antibody response following immunization depend on animal species, inoculated dose, LPS type, route and method of inoculation. The role of LPS as an immunogen in mammals remains controversial. Mice, cattle and rabbits have been readily protected against infection following immunization with LPS.

Abdullabi et al (55) studied the outer membrane proteins (OMP) profile of *P. multocida* type A and the related Taxon 13 strains isolated from bovine pneumonia to determine the heterogenicity of this group and to define a protective immunogen in a mouse septicaemia model by investigation of the immunogenicity model of the OMPs.

An anti – idiotyped strategy was employed. The result showed that polyclonal anti–idiotype antibodies produced could mimic a linear *P. multocida* lipopolysaccehaide molecule. The antilbodies, when used as a vaccine antigen, induced antibodies that recognized lipopolysaccharide (LPS) and imparted acquired protection when syngenic vaccinates were challenged with homologous organisms.

Capsular extract(75,76) has been used to immunize cattle but it could not result in a practical vaccine. Oil adjuvant – type vaccine using capsular extracts obtained by solvent precipitation from the supernatant fluid of fermenter grown *P. multocida* type B has been used for immunization but did not yield encouraging results.

Adjuvant

Administration of many vaccines on their own stimulates a poor host immunological response. This is particularly true of the more recently developed subunit vaccines. An adjuvant is defined any material which enhances the cellular and/or humoral immune response to an antigen. Adjuvant generally elicit an earlier, more potent and longer–lasting immunological reaction against co–administered antigen. The major properties of adjuvant to the antibody response is one or more of the ways shown in Table 4 (77-81).

In addition, the use of adjuvant can often facilitate administration of reduced quantities of antigen to achieve an adequate immunological response. This simplicity consequent economic saving as vaccines (particularly subunit and vector vaccines) are far more expensive to produce than the adjuvant.

A number of different adjuvant preparations have been developed (Table 5). Most preparations also display some associated toxicity and, as a general rule.

Table 4 Major properties of adjuvants

1	They potentiate antibody by increasing the efficiency of antigen presentation and the number of collaborating and secreting cells involved, effectively reducing the optimum required immunogen dose.
2	They enhance immunogenicity, allowing antibody responses to molecules with borderline immunogenic properties.
3	They alter the isotype pattern of antibody responses.
4	They prolong antibody responses by an immunogen depot effect which protects the immunogen from rapid removal and breakdown. Thereby reducing the need for repeated injections.
5	They increase the average avidity and affinity of the antibody response.

Table 5 Overview of the adjuvant preparations that have been developed to date or are under investigation of aluminium. Based substances are the only adjuvants used to any significant degree in humans.

Mineral compounds	Aluminium phosphate ($AlPO_4$)
	Aluminium hydroxide ($Al(OH)_3$)
	Alum ($AlK(SO_4)_2 12H_2O$)
	Calcium phosphate ($Ca_3(PO_4)_2$)
Bacterial products	*Mycobacterial species*
	Mycobacterial components (e.g. trehalose dimycolate muramyl dipeptide)
	Corynebacterium species
	Bordetella pertussis
	Lipopolysaccharide
Oil – based emulsions	Freund 's complete/ incomplete adjuvants(FCA FIA)
	Starch oil
Saponins	Quil A
Liposomes	
Immunostimulatory complexes (ISCOMs)	
Some cytokines	Interleukins 1 and 2

Adjuvanticity, the more toxic it is likely to be. A few different adjuvants may be used in veterinary medicine however (for safety reason), aluminium – based products are the only adjuvants routinely used in human medicine. Application of many of the aggressive adjuvant materials is reserved for selected experimentation purposes in animals.

The concept of enhancing the immune response against an antigen by co – administration of immunostimulatory substance dates back to the beginning of the 20^{th} century. Oil – based emulsion were used from 1916 on. While in the mid – 1920s scientists discovered that the immunological response to administration of

tetanus and diphtheria toxin was increased by co-administration range of (some what unlikely) substances including agar, starch, oil, saponin, tapioca, and bread crumbs.

Few of these substances remain in medical use owing to unacceptable side effects. An ideal adjuvant should display several specific characteristics. It should

- be safe (no unacceptable local systemic responses)
- elicit protective immunity even against weak immunogens.
- be non – pyrogenic
- be chemically defined (facilitates consistent manufacture and quality control testing)
- be effective in infants / young children
- yield stable formulation with antigen
- be biodegradable.
- be non – immunogenic itself.

Adjuvant mode of action. Adjuvants are heterogeneous family of substances, both in terms of their chemical structure and their mode of action. The observed adjuvanticity of any such subtance may be due to one or more of the following factors :

- depot formation of antigen. This results in the subsequence show release of the antigen from the site of injection which, in turn, ensures is prolonged exposure to the immune system
- enhanced antigen presentation to the cells of the immune system
- the direct induction of immunostimulatory substances, most notably interleukins and other cytokines.

In addition to the use of adjuvants per se., modification of the antigen may result in increasing its inherent imunogenicity . Such modifications can include

- polymerization of protein antigens (by, for example, reaction with glutaraldehyde or other cross – linking agents). This approach has been successfully adopted with tetanus and diphtheria toxoids
- conjugation of proteins to polysaccharides
- cationization of protein antigens.

Mineral compounds

Alum – precipitated immunogen

The use of potassium aluminium sulphate to precipitate proteins at alkaline pH is a useful method for extending immunogen availability and prolonging the antibody response. When used intramuscularly it is claimed to induce plasma cell accumulation at the depot site. Its side effects, however, are minimal and it is used in man for diphtheria and tetanus toxoid vaccination. Immunogen precipitates can be injected subcutaneously or by the intraperitoneal route as well as into muscles. Its favoured use is in mice, often mixed with a *Bordetella pertussis* suspension and one of its. Major advantages is that it allows fairly rapid dissemination of immunogen without the risks attached to injection of molecules in solution into primed animals which can lead to rapid fatal anaphylaxis. A commonly used strategy is to divide alum precipitates into very small doses of a few μg and give these every few days over several weeks. Alum – precipitated toxiods and other immunogens are known to induce IgE antibodies in some species.

The only artificial adjuvants widely licensed are aluminium salts, often refered to as “alum”. Aluminum salts are very safe but they do have a number of disadvantages. These include:

1. variations in potency between different batches due to uncontrollable reactions between antigen and the aluminum salt
2. the requirement for refrigeration (complication delivery in developing countries) because they cannot be frozen or lyophilized
3. they occasionally produce abscesses or nodules
4. failure to work with certain antigens
5. at best their immunostimulating properties are limited, particulary with respect to the production of CMI.

In addition aluminium–based products display poor/no adjuvanticity when combined with some antigens (e.g. typhoid or *Haemophilus influenzae* type b capsular polysaccharides)

Bacterial products as adjuvants

Selected microorganisms have been identified which trigger particularly potent immunological responses. The immunostimulatory properties of these cells has generated interest in their potential application as, adjuvants. Examples include various *Mycobacteria*. *Corynebacterium parvum*, *C. granulosum* and *Bordetella pertussis*. Although some such microorganisms are used as antigens in vaccines, they are considered too toxic to be used solely in the role of adjuvant. Researchers thus have sought to identify the specific microbial biomolecules responsible for the observed immunostimulatory activity. It was hoped that these substances, when purified, might display lesser / no toxic side effects while retaining their immune stimulatory capacity.

Bordetella pertussis

The observed adjuvanticity of *Bordetella pertusis* is largely attributable to the presence of pertussis toxin and lipopolysaccharide (LPS). LPS constituent of the cell envelope of gram negative bacteria. Essentially consists of poly- saccharide moieties to which lipid (lipid A) is covalently attached.

While purified LPS displays potent immunostimulatory properties. It also induces various toxic side effects (Table 6) the most prominent of which is pyrogenicity. These effects render application of LPS as an adjuvant unacceptable. Both its immunostimulatory and toxic properties are mainly associated with the lipid A portion of the molecule. Attempts have been made to chemically, or otherwise alter the lipid A portion in order to ameliorate the observed toxicity.

Table 6 Some characteristic biological effects induced by lipopolysaccharide

Pyrogenicity
Generalized and severe toxicity
Adjuvanticity
Activation of macrophages and granulocytes
Activation of complement

Induction of synthesis of TNF , CSF , IL-1,IFN

Some antitumour activity

Succinylated or phthalylinated LPS displays significant reduction in toxicity (up to 100000 fold) while retain in its adjuvanticity. Acid treatment (0.1 M HCI) of LPS obtained from various *Salmonella* species resulted in the production of an LPS derivative termed monophosphoryl lipid A (MPL). This also displays adjuvanticity with title associated pyrogenicity or toxicity. This alteration of biological activity can also be achieved by removal of some of the fatty acids found in the LPS lipid A region. As LPS is effective in activation both cellular and humoral immune responses.

A suspension of killed *B. pertussis* injected jointly with immunogen is another good method of obtaining a potentiated antibody response, particularly in mice. The toxin is known to enhance both IgG and IgE antibody production .

Trehalose dimycolate and muramyl dipeptide

Fractionation of mycobacteria resulted in the identification of two cellular immunostimulatory components trehalose dimycolate (TDM) and muramyl dipeptide (MDP) both are normally found in association with the mycobacterial cell wall. TDM is composed of a molecule of trehalose, (a disaccharide consisting of two molecules of α – D – glucose linked via and 1-1 glycosidic bond) linked to two molecules of mycolic acid (a long–chain aliphatic hydrocarbon–based acid) found almost exclusively in association with mycobacteria. TDM, while retaining its adjuvanticity, is relatively non–toxic.

The structure of the native immunostimulatory MDPs was found to be N–acetylmuramyl–1–alanyl–D isoglutamine. (N-Acetylmuramic acid is a basic component of bacterial peptidoglycan). Native TDM is a potent pyrogen and is too toxic for general use as an adjuvant. The molecular basis underlining MDP's adjuvanticity remains to be fully elucidated. Administration of MDP is however, known to activate a number of cell types which play direct, or indirect, roles in immune function and induces the secretion of various immunomodulatory cytokines (Table 7).

MDP's adjuvant can be used to replace heat killed mycobacterium species in FC. It is also a potent adjuvant for the antibody response when administered with immunogen in saline where it induces an exclusive IgG1 antibody response in mice . It also promotes antibody responses to synthetic antigen.

A number of derivatives were synthesized in the hope of identifying a modified form which retained its adjuvanticity but displayed lesser toxicity. Some such derivatives, most notably threonyl – MDP, moranyl tripeptide and murabutide, displayed some clinical promise in this regard.

Table 7 Some cell types activated upon administration of MDP. Activation induces synthesis of a range of immunomodulatory cytokines by these (and other) cells.

Cell types activated	Macrophages
	Mast cells
	Polymorphonuclear leukocytes
	Endothelial cells
	Fibroblasts
	Platelets
Cytokines and other molecules induced	Interlenkin 1
	Colony stimulating factors
	Fibroblast activating factor
	B cell growth factor
	Prostaglandins

Threonyl – MDP, for example, has been included in the formulation known as Syntesx adjuvant formulation 1 (SAF – 1). Animal studies suggest that this adjuvant is non – toxic and elicits a good B and T cell response.

Corynebacterium granulosum

The *Corynebacterium granulosum* – derived p40 particulate fraction, is composed of fragments of cell wall peptidoglycan and associated glycoproteins. Its administration to animals results in activation of various elements of immune function while displaying little or no toxic effects. In addition to activation of macrophages, p40 induces synthesis of a variety of cytokines, most notably IL-2, TNF, IFN and IFN not surprisingly, p40 was found to enhance both specific and non – specific resistance to a wide range of pathogens and also was shown to display antitumour activity. Clinical trials in humans appear to confirm many of these observations. P40, or derivatives, therefore may yet play a role in human or veterinary immunization programs.

Oil – based emulsion adjuvants

Freund's Complete Adjuvant (FCA or CFA)

This consists of a mixture of mineral oil, a suspension of heat killed *Mycobacterium butyrinum* or *Mycobacterium tuberculosis*. The emulsifier Arlacel A. Immunogens in solution are emultsified with these components to produce an antigen/water microdroplet phase within the oil phase. FCA greatly enhances and prolongs the antibody response as first described by Freund (81). The emulsion is stable and when injected intramuscularly forms a depot of immunogen which only slowly becomes available. The other major attribute is related to the activity of the mycobacteria, some of whose products act as powerful stimulants for cells of the immune system both at local lymph nodes and in the granuloma that forms around the depot. *M. tuberculosis* is the more potent of the two mycobacterial species used. Much antibody is synthesized within the granuloma and this activity continues so long as immunogen persists, which may be for many years. Because of granuloma formation and the tendency for FCA to render the body's own tissues autoimmunogenic it cannot be used in man (Table 8). It is however , by far the most effective and widely used adjuvant for routine antiserum production in animals to soluble antigens and antigens of emulsified cells. FCA induces an almost exclusive IgG antibody response in the rabbit, a predominant IgG, subclass response in sheep and IgG_2 antibodies in the guinea pig.

Table 8 Toxic effects noted when Freund 's complete adjuvant (FCA) was administered to experimental animals

Inflammation / abcess formation at the side of injection
Pyrogenic effect (fever)
Severe pain
Possible organ damage
Possible induction of autoimmune disease
Hypersensitization
Induction of cancer in some animals under some conditions.

Freund's Incomplete Adjuvant (FIA or IFA)

Water phase immunogen can be emulsified with mineral oil without mycobacteria to provide a depot immunization principle. This also provides as income insense long antibody response as there is only gradual uptake and loss of immunogen from the injection site. However, the response with FIA is commonly found to be less reliable and of lower order – this may be due to the fact that open active granulomas do not develop and persist at the FIA depot site. There may also be differences in the antibody isotype response using FIA exclusively. In guinea pigs the response to soluble proteins is in IgG_1. FIA is commonly used for booster immunizations in subcutaneous sites because of its reduced pathogenic effects.

Liposomes

Liposomes are microscopic vesicles consisting of phospholipid bilayers enclosing an aqueous compartment. They can also be bound by single bilayer membrane (unilamellar liposomes) or may be composed of multiple concentric membrane (multilamellar liposomes) (Figure1). Because phospholipids are amphiphilic substances, they form bilayer vesicles spontaneously when dispersed in an excess of aqueous solution, so that liposomes can entrap both hydrophilic and hydrophobic substances in their vesicles (43). Hydrophilic substances are entrapped within the aqueous compartment while hydrophobic substances are bound into the lipid membrane (see Figure 2).

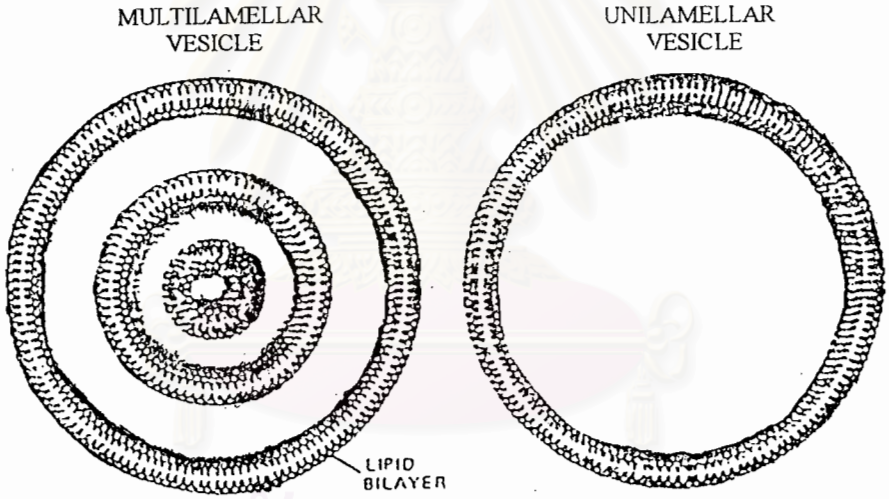


Figure 1 Diagrammatic representation of multilamellar and unilamellar vesicles

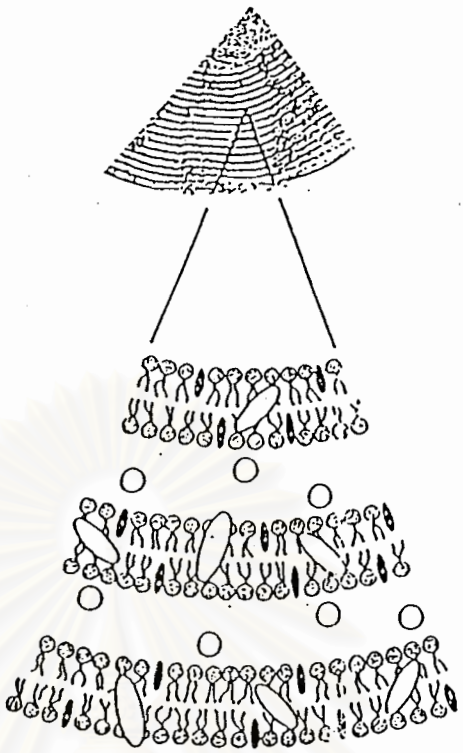


Figure 2 A section of an electron micrograph of multilamellar liposomes shows lipid bilayers alternating with electron opaque aqueous channels. Three of these bilayers are enlarged schematically to illustrate their molecular organization, in which polar heads of phospholipids face the water phase and acyl chains form the hydrophobic regions.

O, open circles denote water soluble drug entrapped in the aqueous channels.

filled oblong shapes are cholesterol

open oblong shapes are lipid soluble drug entrapped in the membrane.

1.Historical background

In 1965, the English scientist Alec D. Bangham made a chance discovery. Introducting water into an erlenmeyer flask containing a thin layer of phospholipid molecules makes these assume an ordinate structure now known as liposomes (43). In the first time, liposomes had been studied as a biological membrane model (42,44). In 1971, liposomes had been extensively studied as drug delivery system (45); one aspect of the studies involved the entrapment of enzymes which could then be administered intravenously to patients for the treatment of inherited storage diseases (46). Liposomes have an advantage as a drug delivery system because they were formed from natural molecules which can be metabolized in the body. Because of the versatility of liposomes structure and ability to incorporate almost any drug regardless of solubility, the use of liposomes as carrier vesicles has been extended to many hundred of drugs. They included chelating agent, antibiotics, drugs, which particular emphasis on anti-tumor drugs, peptide hormones, other proteins and genetic materials (47-58).

2. Chemical constituents.

Chemical constituents of liposomes consist of structural components and non structural components. The structural components are phospholipids and sterols which determine properties such as membrane fluidity, charge density, and permeability. The non structural components are not important components for structural construction of liposome. They were incorporated for some special purpose (59).

2.1 Phospholipids.

Phospholipids are the major structural components of liposomes. They are not soluble but dispersible in water (60). Phopholipids are polar lipids, their molecules consist of a hydrophobic “tail” (the pair of fatty acids) and a hydrophilic polar “head” (the phosphate group) (Figure3) . The backbone of most common polar lipids is either an alcohol such as glycerol or a sphingosine. Corresponding lipids are thus called glycerides or glycerophosphatides and sphingophosphatides (61).

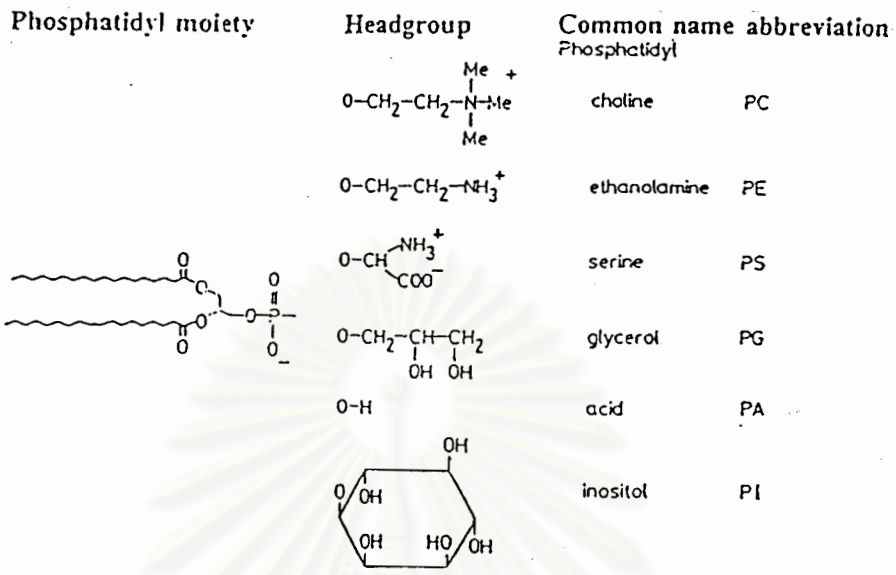


Figure 3. Structure of glycerol containing phospholipids

The most common phospholipids are phosphatidylcholine (PC). They are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar headgroup. In aqueous media they align themselves closely in plannar bilayer sheets in order to minimize the unfavourable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form close sealed vesicles. Phosphatidylcholines contrast markedly with other amphipathic molecules (detergents, lysolecithin) in that bilayer sheets are formed in preference to micellar structures. This is thought to be because the double fatty acid chain gives the molecule an overall tubular shape, more suitable for aggregation in plannar sheets compared with detergents with a polar head and single chain, whose conical shape fits nicely into a spherical micellar structure (Figure 4) . Surfactant molecules, such as dioctadecyl ammonium chloride (DODAC), containing

two hydrocarbon chains (as well as single chain fatty acids, e.g. oleic) can, under certain circumstances, also form bilayer membrane vesicles (59).

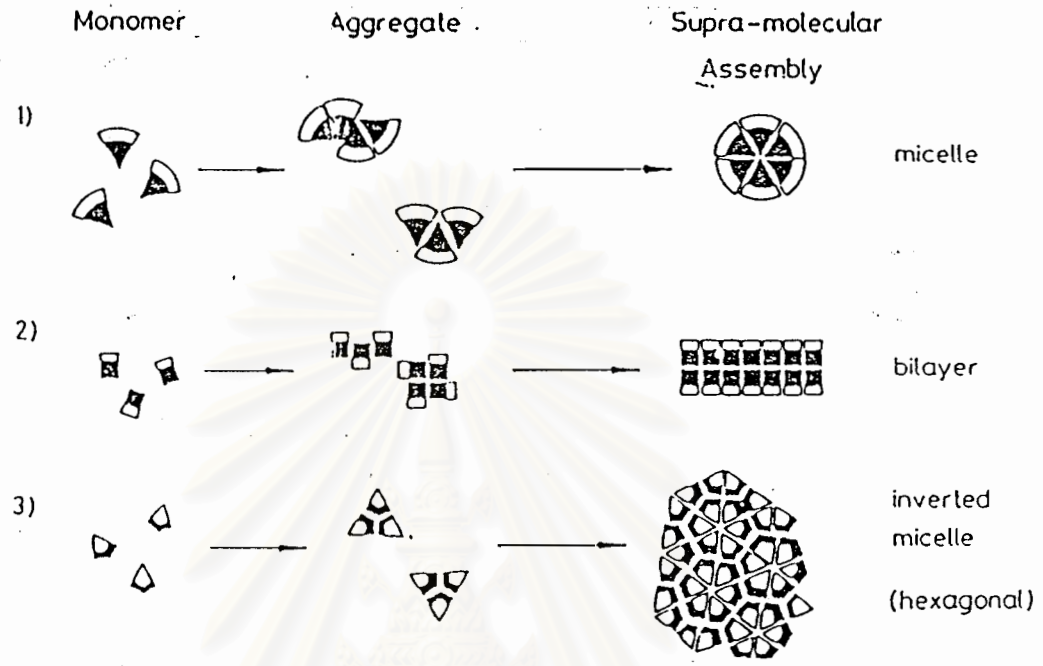


Figure 4. Association patterns of amplhiphiles. Relation between molecular shape and structure of aggregates.

Phosphatidylcholines, also known as "lecithin" , can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean, but less readily from bovine heart and spinal cord. Forming as they do the major phospholipid component of many cell membranes, they are often used as the principal phospholipid in liposomes for a wide range of applications; both because of their low cost relative to other phospholipids, and their neutral charge and chemical inertness. Lecithin from natural sources is in fact a mixture of phosphatidylcholines, each with chains of different length and varying degrees of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contain a higher proportion of fully saturated chains (62).

At different temperatures, lecithin membranes can exist in different phases. In low temperature (T< Tc) ,- gel phases, the hydrocarbon chains are in an

orientationally well ordered state in which the hydrophobic molecular segments are nearly completely in an all-trans configuration; their extension is thus close to the possible maximum. At higher temperatures (T>Tc), however, such high chain order is lost, owing to the orientational chain excitations. This results in a cooperative, first order chain – melting (order – disorder, gel to fluid). Transitions from one phase to another can be detected by physical techniques as the temperature is increased. The most consistently observed of these phase transitions is the one occurring at the highest temperature, in which the membrane passes from a tightly ordered "gel" or "solid" phase, to a liquid – crystal phase at raised temperatures where the freedom of movement of individual molecules is higher. The most widely used method for determining the phase transition temperature (T_c) is differential scanning microcalorimetry (63-64).

An understanding of phase transitions and fluidity of phospholipid membranes is important both in the manufacture and exploitation of liposomes, since the phase behaviour of a liposome membrane determines such properties as permeability, fusion , aggregation, and protein binding, all of which can markedly affect the stability of liposomes, and their behaviour in biological systems.

Binary mixtures of synthetic lecithins of different chain lengths give a main transition intermediate in temperature between those of the individual components, unless the chain lengths are very different, in which case two separate transitions are observed ; in the temperature region between the transitions solid and fluid phases may co-exist, each enriched in one or the other of the components (63-64).

2.2 Sterols

Sterols are apolar lipids (The other apolar lipids, such as paraffins, waxes, fatty alcohols , simple fatty acids, di – and triglyceride, certain steroids, etc.) normally, they do not interact with water or hydrate only weakly because of the lack or too small number of hydrophilic residues (59) . Sterols are important components of most natural membranes, and incorporation of sterols into liposome bilayers can bring about major changes in the properties of these membranes. In mammals, the predominant sterol is cholesterol, with a significant quantity of 7 – dehydro – cholesterol being found in subcellular membranes. Commonly encountered plant sterols are stigmasterol and stiosterol, while ergosterol is an endogenous sterol of yeasts, fungi, and some protozoa. The structure of some sterols were shown in Figure

5, features common to all of these sterols are a 3β - hydroxyl group, a planar steroid nucleus, and an aliphatic side chain, all of which are essential for the characteristic behaviour of sterols in membranes to be displayed.

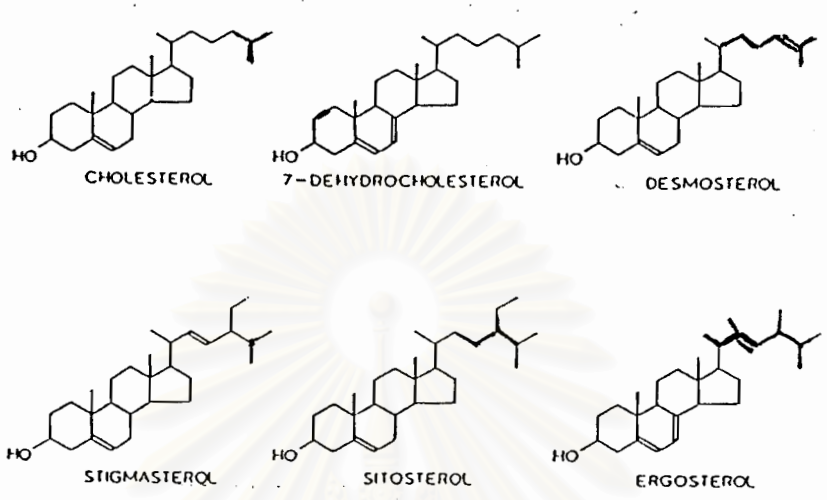


Figure 5. Structure of major sterols found in natural membranes

Cholesterol does not by itself form bilayer structures, but it can be incorporated into phospholipid membranes in very high concentrations-up to 1:1 or even 2:1 molar ratios of cholesterol to PC. In natural membranes, the molar ratio varies from 0.1 – 1, depending upon the anatomical and cellular location. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. The 3β - hydroxyl group is positioned level with the carboxyl residues of the ester linkages in the phospholipids, with very little vertical freedom of movement. The presence of the rigid steroid nucleus alongside the first ten of carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbon towards the terminal end of the chain (Figure 6).

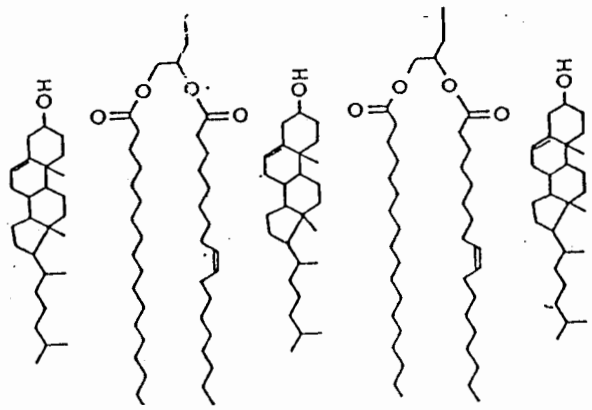


Figure 6. Position occupied by cholesterol in the membrane bilayer.

Cholesterol rests in the membrane with the hydroxyl group on a level with the acyl chain carboxyl groups, and the planar steroid nucleus is thus located parallel with the first nine carbon atoms of the acyl chain, which is the portion of the chain where motion is most severely restricted in the absence of cholesterol. In contrast, motion of the carbons later in the chain is relatively free, and is increased in the presence of cholesterol, as insertion of the steroid nucleus at the head of the chain creates more space for the end carbons in the chain to move.

Above a certain concentration of cholesterol, the membrane area occupied by the acyl chains and sterol combined is greater than or equal to that taken up by the phosphatidylcholine headgroup , so that PC membranes with high levels of cholesterol do not show the chain tilt that is observed in the gel phase of liposomes composed of pure PC.

Addition of cholesterol to PC membranes has a marginal effect on the position of the main transition temperature (Tc): in dipamitoyl phosphatidyl choline (DPPC) the Tc change from 41°C to 44 °C with 33 mol% cholesterol. With increasing concentration, however, cholesterol is able to eliminate evidence of a phase transition altogether, reducing the enthalpy of phase change to zero at 50 mol% (1:1 ratio) , and in so doing altering the fluidity of the membrane both below and above the phase transition temperature.

Cholesterol has relative little effect on the position of the phase transition, but is able to abolish completely the heat of transition. As the concentration of sterol reaches equimolar with phospholipid, the freedom of molecular motion above the

phase transition is decreased, while below the phase transition mobility is actually increased.

At temperature higher than transition temperature, the phospholipid are pushed apart, the packing of the headgroups is weakened, and the fluidity is increased. Below at the transition temperature, the reduction in freedom of acyl chains causes the membranes to condense, with a reduction in area, closer packing and a decrease in fluidity. These changes in fluidity are paralleled by changes in permeabiility of the membrane. By high cholesterol, the permeability of membrane decrease at temperatures higher than the Tc but increase at lower temperatures.

2.3 Non – structural components

For structural purposes, no components other than phospholipids and sterols need to be incorporated into the membrane because the membrane interior is a very fluid aliphatic medium composed of molecules associated by non-covalent interactions it will readily accept and retain a wide range of lipophilic compounds without the need for any fixed chemical structural specificity. Under normal circumstances, these compounds can probably be accommodated in the membrane to a concentration of about 1-10% by weight without serious disruption of the basis bilayer structure, although the membrane integrity as determined by fluidity or permeability may will be altered. In particular cases, where a specific interaction is known to occur between the compound and other membrance components (e.g. fatty acids, ∝-tocopherol) , concentrations higher than 10% may be achieved. Conversely, relatively low concentrations of certain substances such as some polyene antibiotics, will completely disrupt the membrane, as a result of specific interactions.

3. Physical structure

Physical structure of liposomes, such as vesicle size, shape and lamellarity are an important factors affecting physicochemical stability, encapsulation efficiency, tissue distribution, and in vivo circulation lifetimes (65). In the study of the interaction of liposomes with cells in culture have shown that the rate and site of uptake in vivo of small and large liposomes may be quite different. For these reasons,

many workers have aimed to prepare homogeneous suspensions of small liposomes (66).

Physical structure of liposomes are influenced by chemical components and preparing method. Equilibrium liposome radius is determined by the lipid packing properties (67). Chain length plays a much smaller role than the properties of lipid heads, but chain packing parameters do matter (68,69). Addition of cholesterol, for example, results in greater average vesicle size (70). The classification of liposomes according to size is the most common index of charcterization in current use (59).

3.1 Factors Affecting Drug Entrapment(71)

The amount and location of a drug within a liposome is dependent on a number of factors. The location of drug within a liposome is based on the partition coefficient of the drug between aqueous compartments and lipid bilayers, and the maximum amount of drug that can be entrapped within a liposome is dependent on its total solubility in each phase. For example, very little 6- mercaptopurine can be encapsulated in lipsomes because this drug has limited solubility in both polar and nonpolar solvents. The total amount of liposomal lipid used and the internal volume of the liposome will affect the total amount of nonpolar and polar drug, respectively, that can be loaded into a liposome. Efficient capture will depend on the use of drugs at concentrations that do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for nonpolar drugs). The method of preparation can also affect drug location and overall trapping efficiency.

Incorporation of drugs that have intermediate partition coefficients (significant solubility in both the aqueous phase and the bilayer) may be undersirable. If liposomes are prepared by mixing such a drug with the lipids, the drug will eventually partition to an extent depending on the partition coefficient of the drug and the phase volume ratio of water to bilayer. Also , the rate of partitioning will be a function of its diffusivity in each phase. Release rates (a measure of instability) are highest when the drug has an intermediate partition coefficient. Bilayer/aqueous compartment partition coefficients are usually estimated by determining their organic solvent/water (e.g., octanol/water) partition coefficients. They can also be determined precisely by a method described by Backouche and Gerlier, which is based on the physical separation of the aqueous and bilayer phases by

ultracentrifugation after mechanical (ultrasonics at low temperatures) disruption of the liposomes followed by analysis of each phase for drug.

3.2 Size and Size Distribution

The average size and size distribution of liposomes are important parameters with respect to physical properties and biological fate of the liposomes and their entrapped substances. There are a number of methods used to determine this parameter, but the most commonly used methods are the following.

3.2.1 Light Scattering A variety of techniques are available to size liposomes based on light scattering. The popularity of this method depends on its ease of operation and the speed by which one can obtain data. The newer instruments are based on dynamic laser light scattering.

If the liposomes to be analyzed were monodisperse, light scattering would be the method of choice; unfortunately, most preparations are heterogeneous, and they require an accurate estimation of their size-frequency distributions. Light scattering methods rely on algorithm to determine particle size distributions, and the results obtained can be very misleading. Some complex algorithm have been developed in an attempt to deal with this problem. Furthermore, such mehods cannot distinguish between a large particle and a flocculated mass of smaller particles. Most important, it may be necessary to remove any micron-sized particles that are present in the same prior to analysis

3.2.2 Light Microscopy This method can be used to examine the gross size distribution of large vesicle preparation such as MLVs. The inclusion of a fluorescent probe in the bilayer permits examination of liposomes under a fluorescent microscope and is a very convenient method to obtain and estimate of at least the upper end of the size distribution.

3.2.3 Negative Stain Electron Microscopy This method, using either molybdate or phosphotungstate as a stain, is the method of choice for size distribution analysis of any size below 5 μm. It should be used to validate light scattering data that will ultimately be used for quality assurance. For accurate statistical evaluation (+/-5%) , one should count at least 400 particles and not rely on a single specimen for counting.

3.2.4 Freeze Fracture Electron Microscopy This method is especially useful for observing the morphological structure of liposomes. Since the fracture plane passes nonmidplane fractures, the observed profile diameter depends on the distance of the vesicle center from the plane of the fracture. Mathematical methods have been devised to correct for this effect.

For all the microscopy procedure , one should always be on the lookout for aggregated particles or flocs .

3.2.5 Cryoelectron Microscopy This is a relatively new technique that allows direct observation of quickly frozen samples without any staining and is, therefore, the least prone to artifacts. Numerous tests have shown that very quick freezing can preserve the structure, while it may give rise to unreal size distributions due to the fact that larger particles are excluded from the thin (0.2-0.4 μm) film of ice on the microscopic grid.

3.2.6 Gel Chromatography Since the introduction of large pore size gel (Sephacryl S 1000), an easy and quantitative determination of liposome size distribution is possible. In contrast to all other techniques, this method gives a true (i.e., "fit – independent") distribution according to their true hydrodynamic radius for liposomes smaller than 0.3-04 μm .

3.3. Phase Behavior of Liposomes

An important feature of membrane lipids is the existence of a temperature – dependent reversible phase transition, where the hydrocarbon chains of the phospholipid undergo a transformation form an ordered (gel) state to a more disordered fluid (liquid crystalline) state. These changes have been documented by freeze – fracture electron microscopy but are most easily demostrated by differential scanning calorimetry.

The physical state of the bilayer profoundly affects the permeability, leakage rates, and overall stability of the liposomes. By proper admixture of bilayer forming materials, one may design liposomes to "melt" at any reasonable temperature. This strategy has been used to deliver methotrexate to solid tumors. Which are heated to the phase transition temperature of the custom – designed liposomal phospolipids. The phase transition temperature can be altered by using phospholipid mixtures on by adding sterols as cholesterol. The Tm -value can give

important clues as to liposomal stability and permeability and as to whether a drug is entrapped in the bilayer of the aqueous compartment.

4. Preparation of Liposome by Double Emulsion method

Multivesicular liposome (MVLs)

This is a variation of the double emulsion method described above, but which gives rise to liposome of a type which can be produced by no other method. The novelty lies in the fact that the proportions of lipid, solvent composition and duration of shaking are adjusted in such a way that instead of just one single water droplet being contained within the 'oil' droplet of the double emulsion, several water droplets are enclosed. Upon removal of the solvent by evaporation, these water droplets remain intact and form multiple comparments within a single liposomes. Like ordinary double emulsion vesicle, these have an entrapment yield of 50 % or greater. Although no applications have yet been reported for MVLs which could not be performed by normal LUVs, it is conceivable that one may want to deliver to cells or organs of the body number of different agents simultaneously which normally are not very stable in each other's presence. Entrapping the materials in separate comparments of the liposomes could overcome this problem. The size of liposome range from 5 to 30 microns in diameter, depending on the duration of shaking employed from to produce the second emulsion. Shaking for 10 sec is found to be optimal from the point of percentage entrapment, prolonged shaking presumably resulting in rupture and leakage of the internal aqueous comparment to the outside.

Interaction of liposome with the biological element (112)

Since 1970 when liposomes were first proposed and tested in animals as a drug carrier, much of their behaviour within the biological element and ways to control such behaviour have become known. In this respect, relevant to most applications, including immunoadjuvant activity in vaccine, is the effect of biological fluids(with which liposomes come into contact on injection) on, firstly, the liposomal structural integrity and, secondly, the rate at which vesicles are cleared from the site of injection and distributed in tissues. With all these events, liposomal behaviour is determined by the structural characteristics of the vesicles. It is known, for instance, the plasma high density lipoprotein (HDL) remove phospholipid molecules from conventional liposomes (i.e. those made of egg phosphatidylcholine;PC) injected intravenously. Liposomes attacked by HDL become very leaky or disintegrate and release their drug contents. By substituing PC with ‘ high melting ‘ phospholipids such as distearoyl phosphatidylcholine(DSPC) or sphingomyelin, or supplementing phospholipids with excess cholesterol, vesicle bilayers become rigid at 37°C (the body’s temperature) or have their phospholipid molecules packed and thus resist phospholipids loss or HDL. Liposomal integrity is therefore preserved and entrapped solutes (e.g. antigens) remain with the carrier for longer periods of time.

It has been observed that liposome stability (in terms of solute retention) in vivo is directly related to their rate of clearance from the blood circulation (i.e. the more stable the vesicles clearance from the blood circulation (i.e. the more stable the vesicles are the longer they circulate in the blood). The relationship between vesicle clearance and vesicle stability is abolished when a negative or a positive surface charge is imposed on the bilayer surface. For instance, even the most stable, negatively charged liposomes exhibit short half-lives. A similar reduction in the half-life of stable liposomes also occurs as their size increase. Recently, several groups have shown that circulation time of liposomes can be prolonged significantly by rendering their bilayer surface highly hydrophilic , for instance, by the use of polyethyleneglycol covalently coupled to a liposomal phospholipid component. Not surprisingly, liposomes, which exhibit extented half-lives either because of their lipid composition or because of a highly hydrophilic surface are intercepted by the reticuloendothelial system (RES) much more slowly. Obviously, such long-lived

liposomes are especially amenable to targeting to cells with which they do not normally interact in vivo. This is usually achieved by the anchoring of cell-specific ligands(e.g. antibodies and certain glycoproteins and glycolipids) onto somes with cells, their uptake(when it occurs)proceeds though endocytosis, although fusion may be involved to some extent. Interestingly, recent work , has shown that the lysosomotropic pathway of liposomal entry into cells can be interfered with. This is achieved(in vitro) by the use of liposomes which, owing to their lipid composition, can fuse with the vacuoles can fuse with the lysosomes. Thus, it now appears possible to deliver antigens and drugs into more than one cell compartments.

Much of what is known of the fate of liposomes injected intravenously, also concerns preparation administratered by alternative parenteral routes (e.g. intraperitoneal, subcutaneous or intramuscular). For example, a proportion of liposomes, determined by their size, lipid composition and route of injection, enters the lymphatic and, subsequently, the blood circulation. Once there, and in terms of clearance rates, they behave as if given intravenously. On the other hand, whereas liver, spleen and bone marrow intercept most of liposomes given the intravenous route, they will account for only a small fraction of the dose administered by other routes. Much of the remainder(e.g. for liposomes injected subcutaneously or intramuscularly) is retained at the site of injection or ends up in the lymph node draining the injected site. Uptake by lymph nodes is, infact, much greater (over 100-fold in terms of percent uptake per gram tissue) than with any of the other RES tissue.

Work by several laboratories aimed at establishing whether liposomes given by the intragastric route facilitate the absorption of drugs and proteins which are either unabsorbable by or unstable in the gut, have given inconclusive results. In spite of evidence that agent such as tubocurine, insulin, factor VIII, anticoagulants and vitamins reach the blood circulation when given in the liposome form, their absorption is not only minimal but also unpredictable. It is nontheless apparent that liposome of a lipid of composition which renders them resistant to bile or phospholipase attack, protect agent the hostile gut element. It is conceivable that some of the surviving liposomes enter the lymphatics. The ability of liposomes to induce CMI is probably one of their most important features as an immunoadjuvant. Evidence for this has been provided by positive DTH reactions, the lymph node lymphocyte proliferative response test and the induction of cytotoxic T lymphocytes. It is unlikely however, that liposome-induced CMI occur as a result of the antigen-

depot mechanism: adjuvants such as oil emulsion and alum action in this way, induce only or predominantly HI. A more plausible explanation of liposome-induced CMI is related to the presentation of antigen in a hydrophobic microenvironment. It is known for instance, that proteins conjugated to lipids induce DTH in proportion to the lipid's hydrophobicity.The latter, in turn, increases uptake of the complex by macrophage thus improving antigen presentation to T cells. These event may also be favoured by the efficient(and selective) localization of liposomal antigen into the regional lymph nodes.

Liposome immunological adjuvant in vaccines (77)

Immunological adjuvants are agents that can provoke potent immune reponses to antigens. They include aluminium hydroxide (alum), saponins complexed to membrane protein antigens (immune stimulating complexes). Pluronic polymers with mineral oil, killed mycobacteria in mineral oil (Freund's complete adjuvant, FCA), bacterial products such as lipopolysaccharide (LPS) and muramyl dipeptide (MDP), and liposomes. Adjuvants appear to function by one or both of the following mechanisms.

Materials with adjuvant activity and possible sites of action

Activites	**Materials**
Delayed release of antigen	Depot formers, water in oil ; oil in water emulsions ; controlled release devices ; inert carriers (eg, alum)
Mobillization of T-cell help	Proteins as carriers Polyclonal activators of T cells : PPD , poly A : poly U
Modulate Ig receptors on B cells	B–cell mitogens, antigen–polymerizing factors
Localization of antigen in T – dependent areas	Hydrophobic antigens ; addition of lipid tail to proteins

Stimulation of antigen – presenting cells	MDP and derivatives : LPS , lipid A
Facilitate cell – cell interaction	Surface – acting materials ; saponin , lysolecithin, Quil A , liposomes, pluronic polymers.
Focusing of antigen on leukocytes with Fc receptors	Alternate pathway of complement activatior : inulin , zymosan, endotoxin
	LPS,lipopolysaccharide;MDP, muramyl Dipeptide; PPD, purified protein derivative

The first mechanism involves creating a depot (as , for example, by alum and oil emulsions) at the site of injection, which prolongs the release and interaction of antigens with antigen – presenting cells (APC). The APC may also invade the depot area in the presence of local inflammation, and adjuvant antigen complexes may migrate to area in the regional lymph nodes containing T cells. The second mechanism is activation of macrophages (for example LPS, MDP) which release interleukin 1 (lL –1). The combined action of IL – 1 and antigen on T cells produces interleukin 2 (IL –2) and other mediators which activate effector T cells (cell – mediated immunity , CMI) or antibody – forming B cells (humoral immunity , HI). Some of these factors also promote the retention of circulating lymphocytes in the lymphoid tissues adjacent to the site of injection. It has been suggested that strong and persistent immune responses may be elicited by targeting antigens to interdigitating cells and follicular dendritic cells which, unlike macrophages, constitutively express major histocompatibility (MHC) class II antigens . As these specialize in presenting antigen to T and B lymphocytes respectively. CMI and HI could be favoured selectively. Of the adjuvants mentioned, only alum is licensed for use in humans. But it is far from ideal : it is not always effective. It increases CMI only slightly if at all and, as it connot be lyophilized, it requires refrigerated storage. Many of the other adjuvants are toxic, inducing granulomas , acute and chronic inflammations (CFA) , cytolysis (saponins , some Pluronic polymers) and pyrogenicity (LPS and MDP). However with some of the agents, toxicity can be curtailed without loss of adjuvanticity through changes in the adjuvant's structure (LPS , MDP) or through the choice and synthesis of appropriate adjuvant analogs

(saponin, Pluronic polymers). Concerted efforts are now in progress to develop safe and effective adjuvants to meet the challenges of subunit and peptide vaccines and new insights into the ways in which immunlty is produced. An ideal adjuvant should comply and certain criteria which, in addition to some practical ones (for example inexpensive raw materials , simplicity of manufacture, stability in storage – preferable in a freeze – dried form). Would also include a biodegradable, non – toxic, and non – immunogenic nature, the ability to elicit both CMl and Hl to antigens administered by a variety of routes, synergistic action with other adjuvants if needed , and a potential for selective interaction with populations of immunocompetent cells.

Adjusting the proportions of each polymer varies the rate of release of the entrapped antigens. Entrapped antigens can be released at predetermined rates or intervals after a single immunization and so may obviate the need for booster doses of a vaccine. The size of the microparticle is critical. Particles with a diameter of less than 10 microns are taken up by macrophages and transported to the draining lymph node and produce a more rapid rise in antibody levels compared with larger (> 10 microns) particles that remain at the injection site, comparable with the suitable in CFA emulsion for antigens is presented schematically in figure 7.

Both the activation of T cells and of B cells is important for an effective imunogenic reaction, because, in a complex way, they interact with one another, either directly or through interleukins. The T-helper (T_H) cells are the “principal orchestors” of the immune response because they are needed for the activation of the major effector cells in the activation of the major effector cells in this response (i.e. cytotoxic T (T_c) cells and antibody-producing B cells). Antigen presentation is mediated by specialized macrophages (APC). After internalization of the antigen-loaded particle or free antigen, immunorelevant epitopes are presented on the surface of the APC, in combination with a major histocompatibility complex (MHC), the T_H cells are attracted and activated by two signals: binding of the T-cell antigen receptor to the MHC complex and production of interleukin-1 by the APC. The activated T_H cells trigger a complex cascade. They release lymphokine (a) stimulating B lymphocytes to proliferate and produce specific antibodies after differentiation as well as memory cells, and (b) activating granulocytes, macrophages, and natural killer cells. Cytotoxic T cells are activated directly. Moreover some long-lived T_H cells seem to provide a memory function in the T-cell compartment of the immune system.

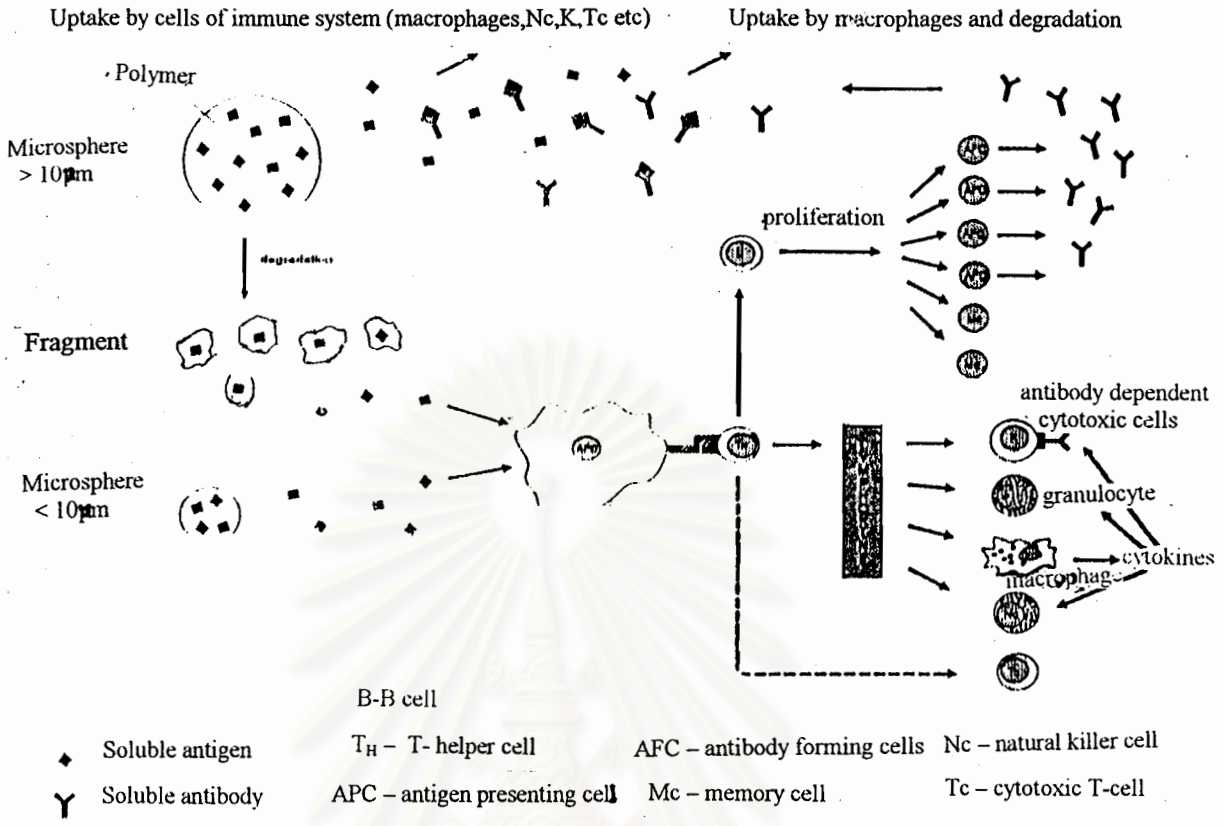


Figure 7 Interaction of particulate carrier system with the immune system.

Certain molecules appear to trigger B lymphocyte directly (thymus-independent antigens). This mechanism is still incompletely directly understood because the antibodies produced are mainly of the IgM class, and little or no immunological memory is affected.

The release of the antigen from microparticle is controlled by a variety of factors, such as microparticle morphology, microparticle size, and the polymer used for microencapsulation. In this selection we will attempt to summarize some of in vivo results obtained with experimental vaccine-delivery system after parenteral application, although one has to bear in mind, that such a retrospective comparison is obscured by the unknown effect of experimental differences on the biological response.

For one, different species were used (e.g. mice, rats, and guinea pigs). Injection site and vehicles for microparticle administration were different, such as IP,

IM, or SC. Therefore, it is not surprising reaction varied considerably from study to study. Second, there is no consensus on a generally accepted standard dose for the respective animal model used. Only two groups reported afforts in establishing an adequate dose for tetanus toxoid in mice. A dose-response relation for microencapsulation antigens has been established to our knowledge; therefore, interspecies comparisons and in vivo-in vitro correlation of antigen release from animal studies are subjected of speculation. Third, and most importantly, there seems to be little agreement in the literature on a standard immunization protocol for in vivo studies. Positive and negative controls, time schedule for boostering, and time intervals for blood sampling differ considerably from group to group.

After subcutaneous primary and secondary immunization of mice with ovalbumin (OVA) in microparticles (5.3 microns) . The antibody titers reached were comparable to those reached by immunization with OVA in FCA over 12 – week period.

Microparticles (< 10 microns) were taken up by macrophages and transported to the draining lymph nodes. The researchers also demonstrated that different sizes of microparticles could be administered in combination to provide a biphasic antibody response. In this review liposomes are discussed as attractive adjuvant candidates**.**

It is fairly clear that the adjuvant property of liposomes is a physical rather a chemical effect. When injected intravenous liposomes are taken up mainly by the liver and spleen. Interestingly, intravenous administration is a very poor method of immunizing mice with material in liposomes intraperitoneal and subcutaneous administration being routes with lead to a greater immune response. It seems likely that liposomes injected by these two routes will remain at the site of injection for a long period. Thus, liposomes may well exert their adjuvant effect by the same method as many other particulate adjuvant that is, by retaining a "depot" of antigen at the site of injection. The phagocytosis of liposomes by cells such as macrophages may also be an important factor. This suggested by the fact that the incorporation of more than 30 mol% cholesterol into liposomes markedly reduces the immune response to the antigen entrapped within them. This level of cholesterol has also been shown to reduce the rate of digestion of liposomes by macrophages.

The research group of Naito et al, 1996 (113) immunized adjuvant liposome and alum in rabbits. Antibody response after immunization with surface – coupled ovalbumin (OVA) of liposomes was investigated in mice. OVA was coupled to the

surface of liposome via amino groups using glutaraldehyde. OVA – liposome conjugate induced a significant anti – OVA IgG antibody production in mice. However, no IgE antibody production specific for OVA was observed. Immunization with OVA – liposome induced IgE–specific unresponsiveness even after the subsequent challenge with OVA adsorbed with aluminium hydroxide (OVA – alum), which induces a high level of IgE antibody production. Furthermore, following the primary immunization with OVA – alum. A secondary challenge with OVA – liposome boosted anti – OVA IgG but not anti – OVA IgE antibody production. These results show the potential of the antigen – liposome conjugate for the development of a vaccine with the least allergic reaction and also for the application of immunotherapy.

Since Allison and Gregoriadis 1974 (83) demonstrated that liposomes could enhance the antibody response to diphtheria toxoid. They have claimed that whilst liposomes enhance the immune response they also prevent any adverse hypersensitivity reaction to the antigen by shielding it from antibodies. They have so far demonstrated for diphtheria toxoid , that liposomal entrapment of antigen prevents arthus type hypersensitivity reactions when the material is injected into the footpad of a primed mouse , and also prevents the death of mice with high circulating antibody when injected intravenously, the role of liposomes as adjuvants in stimulating an immune response has received a great deal of attention. Since that time liposomes have been shown to be effective adjuvants for a large number of protein antigens (see Table 9).

Table 9 Immunoadjuvant action of lipsomes: antigens studied

Diphtheria toxoid	Influenza virus 9 A/PR/8 strain) Reconstituted envelope
Tetanus toxoid	Influenza virus (x –49 strain) rosette
Cholera toxin	Polio virus 3VP2 peptide
Mycobacterium leprae antigens	Foot – and – Mouth disease virus VPI peptide
Neisseria gonorhoeae proteins	Rubella virus (M –33 strain) rosettes
Proteus mirabilis major outer membrane protein and LPS	Adenovirus type 5 hexon

Salmonella typhimurium LPS	
	Encephalomyocarditis virus (inactivated)
Streptococcus sobrinus ribosomal protein	
Streptococcus pneumonia type 3	Semliki Forest virus (inactivated)
Sterptococcus mutans carbohydrate – protein conjugate	*Herpes simplex* virus type 1 antigen
	Epstein – Barr virus glycoprotein
Nippotrongylus brasiliensis antigens	
Leishmania mexicana gp63 and fipophosphoglycan	Rabies glycoprotein
	Gross virus cell suface antigen
Lishmania major soluble antigeas (LV39	*Hepatitis B* surface antigen
Plasmodium falciparum synthetic peptide and J2 311 promastigotes)	*Hepatitis B* surface antigen polypeptide
Plasmodium falciparum merozoite enriched antigen	*Hepatitis B* virus envelope pre – S (120 – 145 sequence) peptide
	Influenza virus (A/PR8/34 strain) glycoproteins
	Fibrosarcoma surface antigens
	Color tumour (LS 17T) membrane vesicles
	Tumour (L_3C) antigens
	Tuberculins (purified protein derivative and peptide)
	Rat colon tumour antigens
	Spematozoal polypeptide fraction
	Nigerian Echis carinatus venum

* Antigens shown are only those of medical or veterinary relevance. Antigens were incorporated by various method into liposomes of different sizes, compositions and other characteristics. In some studies, liposomal antigen formulations also contained other adjuvants.

Liposomes are being investigated in the design of subunit vaccines for viral diseases and are able to efficiently present the surface glycoproteins of many enveloped viruses for stimulation of a protective immune response. The viral glycoproteins are anchored in the liposomal bilayer via a transmembrane segment and assume a conformation analogous to their native conformation in the viral envelope. Influenza virus surface proteins have also been shown by Almeioda et al. 1998(113) to orientate themselves in liposomes in a manner which, under the electron microscope appears similar to their disposition in the intact influenza virus. Moreover the "virosomes" so formed can be agglutinated by anti – influenza antisera. The production of the virosome was prompted by the knowledge liposomes were adjuvants, and the need for a non – pyrogenic influenza vaccine. Whole influenza virus is a good immunogen, but is also pyrogenic. Influenza protein are not pyrogenic, but also are not very good immunogen.. It is hoped that the combination of non – pyrogenic lipid with the subunits should produce an non – pyrogenic, but immunogenic , preparation.

Since Power D.C. et al, 1995 (114) tested cytotoxic T lymphocyte responses to a liposome – adjuvanted influenza a virus vaccine in the elderly. The result of anti – influenza virus CTL activity was enhanced to a significantly greater extent by the liposome vaccine that by the control subvirion vaccine.

An important feature of liposomes as adjuvants is their abillity to induce CMl. This has been shown by positive DTH reactions the lymph node lymphocyte proliferative response test and the induction of cytotoxic T – lymphocytes. Liposome – induced CMI cannot be explained by the antigen – depot mechainsm since adjuvants such as alum and oil emulsions, which act in this way induce only or predominantly HI. Since proteins coupled to lipids are known to induce DTH in proportion to the latter's hydrophobicity, it is likely that the increased internalization of hydrophobic antigen lipids by macrophages ultimately improve antigen presentation to T cells. As with HI, these events may also be favoured by the efficient (and selective) uptake of liposomal antigens into the regional lymph nodes. The adjuvanticity of liposomes is basically a projection of the system's vesicular structure and perhaps of its lipoid nature, rather than of the identity of its lipid components or other secondary characteristics. The latter, however, are known to effectively control the behaviour of liposomes in vivo and may thus be instrumental in the way immunoadjuvant activity is expressed, both qualitatively and quantitatively. Several

groups have, therefore, investigated the extent to which bilayer fluidity. Number of lamellar in bilayers, vesicle size and surface charge, lipid to antigen mass ratio and mode of antigen localization within liposomes influence adjuvanticity . All appeared to have an effect but conclusion as to the role of individual parameters have often been contradictory or have not been confirmed.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1.Microrganisms

Lyophilized *Pasteurella multocida* vaccine strain serotype 8A was obtained from Division of Biological Product, Department of Livestock, Ministry of Agriculture and Cooperation, Bangkok, Thailand

The temperature sensitive *Pasteurella multocida* 2T35 mutant strain deriving from *P. multocida* isolate serotype 8A treated with N-methyl-N-nitrosoguanidine was obtained from Animal Vaccine Research Unit, Chulalongkorn University.

2. Experimental Animals

Rabbits: The immune sera were produced in young adult New Zealand White rabbits weighing about 2 – 4 Kg each.

3. Media

Tryptic soy agar (Merck, Germany)

4. Blood

Sheep blood (Faculty of Veterinary Sciences, Chulalongkorn University)

5. Chemicals

Barbital (BDH Chemicals LTD Poole, England)

Bovine serum albumin (Sigma, U.S.A)

Cholesterol (Sigma, U.S.A)

Citric acid (Mallinkrodt chemical works, England)

Coomassie brillant blue G-250 (Biorad, U.S.A)

Dichloromethane (Merck, Germany)

Goat anti-rabbit IgG enzyme HRP conjugate (Zymed, U.S.A)

Hydrochloric acid (Merck, Germany)

Hydrogen peroxide solution, 35 % (Merck, Germany)

Lecithin (Sigma, U.S.A)

Polyvinyl alcohol (Merck, Germany)

Potassium aluminium sulfate ($AlK(SO_4)_3$) (Fluka, Switzerland)

Potassium chloride (May&Baker LTD Dageham, England)

Potassium dihydrogen phosphate (May&Baker LTD Dagenham, England)

Potassium thiocyanate (Merck, Germany)

Skim milk (Difco, U.S.A)

Sodium barbital (BDH Chemicals LTD Poole, England)

Sodium bicarbonate ($NaHCO_3$) (Wakopurechemical industries, Japan)

Sodium carbonate (Merck, Germany)

Sodium carboxymethylcellulose (Merck, Germany)

Sodium chloride (NaCl) (Carlo Erba, Germany)

Sodium hydroxide pellets (Merck, Germany)

Sodium hydrogen phosphate (Merck, Germany)

Sodium nitride (NaN_3) (Difco, U.S.A)

Sulfuric acid 98 % (Mallinkrodt chemical works, England)

Thimerosol (Keck' s, U.S.A)

Tris-Hydrochloride (Sigma, U.S.A)

Triton X –100 (Amresco, U.S.A)

Tween 20 (Merck, Germany)

Xylene (Mallinkrodt chemical works, England)

6.Instrument

Analytical balance (Sartorious, U.S.A)

Autoclave (Hirayama, Japan)

Automatic pipette (Nomara, Switzerland)

Colony counter 500 (Suntex, Taiwan)

Deep freezer –85 °C (Forma Scientific, U.S.A)

Freezer -20°C (Tropical Ariston, Italy)

Freezedryer (FTS, U.S.A)

Hamilton syringe (Helmilton, Switzerland)

Hot air oven (Precision, U.S.A)

Incubator (Memmert, Germany)

Light microscope (Olympus, Japan)

Larminar flow (ISSCO, Thailand)

Magnetic stirrer (Thermolyme, U.S.A)

Microplate Reader (Biorad, U.S.A)

Micropipette (Labsystem, Finland)

Millipore filter set (Gelman Science, U.S.A)

Multichanel micropipette (Socodrex, Switzerland)

pH meter (Beckman, U.S.A.)

Refrigerator (Hitachi, Japan)

Refrigerated centrifuge (Hitachi, Japan)

Refrigerated microcentrifuge (Sigma, U.S.A)

Shaker incubator (New Brunswick Scientific, U.S.A)

Sonicator (Heat System-Ultrasonic Inc., U.S.A)

Stereomicroscope (Leitz Wetzlar, Germany)

Ultracentrifuge (Beckman, U.S.A)

Vaccuum suction (Gast, U.S.A)

7. Glassware

Beaker (Pyrex, U.S.A)

Erlenmeyer flasks (Pyrex, U.S.A)

Funnel (Pyrex, U.S.A.)

Glass bottle (Pyrex, Germany)

Glass slides (Clay Adam, U.S.A)

Measuring cylinders (Pyrex, U.S.A)

Measuring pipettes (HBG, Germany)

Pasteur pipettes (John poulten, England)

Petri dishes (Pyrex, U.S.A)

Syringes (Nipro, Thailand)

Test tubes (Pyrex, U.S.A)

Volumetric flask (Witeg Diffico, Germany)

Volumetric pipette (Brand, Germany)

8. Other

Aluminium foils (Diamond foil, U.S.A.)

Centrifuge tube plastic 50,500 ml (Nalgene, U.S.A)

Clip (Medi-clip, England)

Dialysis tubing (Pierce, U.S.A.)

Disposable gloves (Imperial, Thailand)

Hydrophilic polypropylene membrane filter 0.45 μm (Gelman, U.S.A)

Microtiter plate (Nunc, Denmark)

Microtube plastics (Treff Lab, Switzerland)

Nitrocellulose membrane 0.2 μm (Micron Separation Inc., U.S.A)

Whatman filter paper No.1 (Whatman, England)

METHODS

1. Calibration curve of standard protein (Assay by Lowry method)

(115,116)

The assay is based on the reaction of protein with an alkaline copper tartrate solution (Reagent A) and Folin reagent (Reagent B). There are two steps which lead to color development: the reaction between protein and copper in alkaline medium, and the subsequent reduction of the Folin reagent by copper treated protein. Protein affects a reduction of the Folin reagent by loss of 1,2 or 3 oxygen atoms, thereby one or more of several possible reduced species which have a characteristic of blue color with maximum absorbance at 750 nm. and minimium absorbance at 605 nm..

The procedure of method could be explained as follows: the working reagent A was prepared by adding 20 μl of reagents to each ml of reagent A. (This working reagent A is stable for 1 week even though a precipitate will form after 1 day. If precipitate forms, warm the solution and vortex.)

Standard solution containing an accurate amount of bovine serum albumin (BSA) 1.35 mg/ml was diluted to 5 dilution 50, 100, 200, 400, 800 μl /ml of BSA. Triplicate , 5 μl of each standard was pipetted into separated wells of microtiter plate. The 25 μl of reagent A was added , subsequently 200 μl of reagent B was added into each well. Then, let the plate mixed for 5 s. by microplate mixer. After 15 min, absorbances can be read at 750 nm.. The plot of the average absorbances versus known concentrations was made. The relation between absorbances and concentrations was determined by linear regression.

2. Preparation of protein extract from *Pasteurella multocida* (117)

2.1 Culture organisms

The culture used for protein extraction was *Pasteurella multocida,* 8A and 2T35. They were thawed from stock cultures (kept at -70°C) and streaked for isolation on tryptose soy agar containing 3 % sheep blood. The culture was incubated at 37°C for 48 hours.

2.2 Preparation of potassium thiocyanate (KSCN) antigen extract

The cells were harvested in 6 ml of 1M potassium thiocyanate (KSCN) in 0.9 % sodium chloride per plate and slowly stirred in shaker water bath at 37°C for 6 hours. This suspension was centrifuged at 4°C to pellet the cells. The supernatant was dialyzed with 0.01 M dialysis solution to remove any possible dialyzable fragments. The dialysate containing protein antigen was collected and concentrated by freeze-drying using lyophilizer (FTS system). It was stored at -20°C until used.

2.3 Protein determination of *Pasteurella multocida* antigen extract

The protein contents of antigen extract were determined by Lowry method as described above.

3. Preparation of immunization antigens

3.1 Preparation of liposome containing protein extract from *Pasteurella multocida* by double emulsion technique.(118,119)

Liposome containing protein extract from *Pasteurella multocida* was prepared by double emulsion technique (modified from the method of Kato, Arakawa and Kondo1985(69) as follows: total lipid mixture in the molar ratio of 1:1 of egg yolk lecithin to cholesterol was dissolved in 10 ml of dichloromethane at 50 mg/ml. Protein extracted from *Pasteurella multocida* was dissolved in phosphate buffer solution (PBS), pH 7.4 and polyvinyl alcohol concentration at 0.5 % (119) to obtain protein concentration at 2 mg/ml. To 10 ml of protein solution, an equal volume of the lipid solution was added, the mixture was vigorously agitated by vortex mixer for 10 min to give a w/o emulsion. The emulsion obtained was quickly added by stirring to 100 ml of 0.02 % sodium carboxymethylcellulose(CMC) in PBS pH 7.4 to yield a w/o/w emulsion. After 10 min. stirring, another 100 ml of 0.02 % CMC in PBS pH 7.4 was added to complex emulsion and the stirring was further continued for 10

hours until the dichloromethane completely evaporated. In this way, liposome containing protein extract form *Pasteurella multocida* was obtained.

The aqueous dispersion of liposome was centrifuged at 20000 g for 30 min. The pellet was washed three times with the PBS pH 7.4 by centrifugation and redispersed in 10 ml of the same medium for further analysis.

3.1.1 Percent protein entrapment

The volume of 1 ml liposome suspension was pipetted into microcentrifuge tube. The encapsulated protein was separated from free protein by centrifugation at 20000 g for 30 min. The pellet containing entrapped protein was ruptured by adding 1 ml of 2 % w/v Triton X-100. After incubated at room temperature for 30 min, the clear solution was assayed by the modified Lowry method (Bio-rad DC protein assay). The absorbances were read at 750 nm and compared with the corresponding standard curve (all analysis were done in triplicate). The percent protein entrapment was calculated by following equation.

$$\text{Percent protein entrapment} = \frac{\text{Total protein content in liposome vesicle X 100}}{\text{Initial protein content}}$$

3.1.2 Particle size analysis

Laser particle sizer (Mastersizers long bed Ver 2.11 with 300 mm range lens, 240 mm beam length) was used to determine particle size of liposome. Particle size distribution was analysed by the curve plotted between particle diameter versus percent volume of particles. Cumulative frequency curve was plotted between cumulative percent under size versus particle diameter , and the median diameters were determined.

3.1.3 Determination of dichloromethane residue in liposome(119,120)

Dichloromethane was used as organic solvent to dissolve lecithin and cholesterol in the primary emulsion step for liposome preparation. Dichloromethane is toxigenic halogenated hydrocarbons, and the tentative maximum permissible concentration is not more than 0.05 mg/ml in blood. The other reason for detection of residual dichloromethane is that dichloromethane may induce protein denaturation during the primary emulsification step. The dichloromethane residue was determined by gas chormatography and the principle of the procedure was as follows:

Standard preparation – dichloromethane in hexane at 1 mg/ml

Test preparation – 3 ml of liposome

Chromatograph system – the gas chromatography is equipped with a flame-ionization detector and column packed with 5 % SE-30. The column is maintained at about 50°C, the injection part is maintained about 180°C, and the detection is maintained at 180°C. Dry helium is used as the carrier gas at a flow rate of about 25 ml per minute.

Procedure – The test preparation (standard preparation) was heated at 50°C and injected on head space to the gas chromatograph, and measure the peak response.

The sample was assayed for dichloromethane concentration by Scientific and Technological Research Equipment Centre.

3.1.4 Determination of potassium thiocyanate in protein extract (119)

Accurate volume of 0.1 N $AgNO_3$ solution 40 ml was transferred through glass funnel to 250 ml erlenmeyer flask. Seventy-five millitre of water (halogen-free), 5 ml of HNO_3 (1:1) and 2 ml of Fe alum solution (Ferric ammonium sulfate [$FeNH_3(SO_4)_2 \cdot 12H_2O$] 8 g in water 100 ml) were added. The solution was titrated with thiocyanate solution until the solution is reddish brown, which remains after shaking vigorously for 1 minute. Record buret reading and set flask aside for 5 minutes, shaking occasionally and maintaining end point color, matching with color of reference solution (To mixture of 5 ml HNO_3 (1:1), 2 ml Fe alum solution and 115 ml H_2O, add 0.02 ml 0.1 N thiocyanate, note exact volume used.). From total volume thiocyanate solution used in titration, substract volume contained in reference solution.

$$\text{Normality} = \text{g } AgNO_3 \text{ X } 1000/ \text{ (ml of titer) X } 169.87$$

$$\% \text{ concentration} = \frac{\text{Normality KSCN in protein extract}}{\text{Normality KSCN initial}} \text{ X } 100$$

3.2. Prepraration of alum containing protein extract from *Pasteurella multocida.*(121)

Protein extract from *Pasteurella multocida* was dissolved and diluted in phosphate buffer solution (PBS), pH 7.4 to obtain protein concentration at 1000 μg/ml. The mixture containing 1 ml of protein, 500 μl of 1M sodium bicarbonate, and

1 ml of 0.2 M aluminium potassium sulfate was prepared and vigorously agitated by vortex mixer for 10 seconds.

The aqueous dispersion of alum was centrifuged at 13000 g for 5 minutes. The precipitates were washed three times with the PBS pH 7.4 by centrifugation and redispersed in 1 ml of the same medium.

4. Immunization and determination of immune response

4.1 Experimental design and serum sample collection from rabbits immunized with potassium thiocyanate extract of *Pasteurella multocida*

Rabbits were divided into 8 groups (3 rabbits/group), each was immunized 2 doses, 1 week apart:

Group 1 was immunized with liposome *Pasteurella multocida* 8:A

Group 2 was immunized with liposome *Pasteurella multocida* 2T35 lot A

Group 3 was immunized with liposome *Pasteurella multocida* 2T35 lot B

Group 4 was immunized with alum *Pasteurella multocida* 8:A

Group 5 was immunized with alum *Pasteurella multocida* 2T35

Group 6 was immunized with *Pasteurella multocida* 8:A protein extract

Group 7 was immunized with *Pasteurella multocida* 2T35 protein extract

Group 8 was immunized with phosphate buffer pH 7.4

Blood was collected from the lateral ear vein of each rabbit prior to immunization and every week for 3 months. Serum was separated by centrifugation at 8000 g for 10 min, aliquoted in microtubes and frozen at -20°C until analyzed.

4.2 Determination of antibody titer by enzyme-linked immunosorbent assay (ELISA) (59,122)

Antibody against potassium thiocyanate protein extract was performed as followed: polystyrene 96-well plates were coated with 100 µl/well of *Pasterella multocida* KSCN extract 1 µg per ml in coating buffer, and incubated overnight at 4°C. The KSCN extract was removed. The wells were washed with PBS (pH 7.4) 3 times, blocked with 3 % BSA in PBS for 1 hour and were washed again as above. One hundred microliter of each serum dilution (1:10-1:10^6)in 1 % BSA in PBS was added and incubated in triplicate KSCN extract-coated wells for 1 hour, After the wells were washed with PBS-Tween, 100 µl of solution containing goat anti-rabbit-

IgG-HRP conjugate was added to the wells and incubated for 1 hour at room temperature. After another PBS-tween wash, 100 µl of the substrate (o-phenylenediamine) was added and incubated for 15 minute. The reaction was stopped by adding H_2SO_4, 50 µl per well. The optical density at 492 nm was read by microplate reader and the serum dilution showing OD of 0.5 was determined as antibody titer.

Antibody was also assayed by using the whole cell antigen, *P. multocida* 8A or *P. multocida* 2T35 as coating antigen. The ELISA process was performed by the similar protocol described above. The serial dilution of rabbit immune sera was reacted with homologous and heterologous whole cell coated at about 6x 10^7 CFU/well in microtiter plate and the antibody titers were monitored.

5. Determination of bactericidal activity of immune sera (122,123)

Antibodies were tested for their bactericidal capacity. Triplicate of 20 µl of antibody samples were incubated with 100 µl of *Pasteurella multocida* suspension (about 2 X 10^3 cfu/ml) in modified barbitone buffer for 10 minutes at room temperature in microtiter plates. The plates were further incubated for 30 minutes at 37°C. From each well of triplicate, 20 µl sample was removed and plated on 5% sheep blood agar. The number of colonies (T_{30}) was counted after incubation at 37°C, 24-48 hours. The initial bacterial inoculum was confirmed retrospectively by plate count and was calculated as 0.5 x the mean cfu/ml at T_0. The percentage of killing (K%) was then calculated by the formula:

$$\%K = \left[1 - \frac{\text{Mean cfu /ml at } T_{30}}{\text{Initial bacterial inoculum}(0.5 \times T_0)} \right] \times 100$$

Immune rabbit serum against the whole cell of *Pasteurella multocida* was incubated as positive serum control and modified barbitone buffer was included as negative control.

CHAPTER IV

RESULT AND DISCUSSION

1. Calibration curve of standard protein

In this study, Lowry method was used to determine concentration of protein remaining in liposome vesicles that were ruptured with Triton X-100. The relationship between concentration of standard protein bovine serum albumin (BSA) in PBS 7.4 and absorbance at 750 nm was shown in table 10 and standard curve was shown in Figure 8 with the correlation coefficient of 0.9996.

Table 10 Relationship between concentration of standard protein (BSA) in PBS pH 7.4 and absorbance at 750 nm.(Assay by Lowry method)

Concentration of BSA (µg/ml)	Absorbance
50	0.0765
100	0.094
200	0.126
400	0.197
800	0.325

2. Preparation of protein extract from *Pasteurella multocida*

2.1 Culture of organism

Pasteurella multocida grew on tryptic soy blood agar (TBA) at 37°C about 24 – 48 hours gave white to yellow color colony. The cells were harvested in 6 ml of 1 M potassium thiocyanate (KSCN) in 0.9 % sodium chloride per plate.

2.2 Preparation of potassium thiocyanate antigen extract

The potassium thiocyanate extract was freeze-dried and stored in closed containers, and kept at –20 °C until used. The average concentration of lyophilized protein extract was 141.44±1.53 μg/ml and 139.78±2.01 μg/ml from liquid KSCN extract of *P. multocida* 8A and 2T35, respectively.

2.3 Protein determination of *Pasteurella multocida* antigen extract

The potassium thiocyanate (KSCN) extract of *P. multocida* was a subcellular antigen containing various components such as protein, carbohydrate, lipopolysaccharides (LPS), DNA, and RNA. Since the main component was protein, it was determined by dye-binding method which is a simple and accurate method for determining concentration of solubilized protein. In this study, the average protein concentration from three determinations was 116.50 ± 6.31 μg/mg of *P. multocida* 8A and 82.91 ± 6.04 μg / mg of *P .multocida* 2T35.

3. Preparation of immunization antigen

3.1 Preparation of liposome containing protein extract form *Pasteurella multocida* by double emulsion technique

Liposome containing protein extract from *Pasteurella multocida* was prepared by double emulsion technique based on the method of Kato et al. 1985(67). This technique was selected for preparing liposome containing protein extract from *P. multocida* because of several benefit factors. The first, it enables the polymer to become associated with surface of each bilayers in the multilamellar structure, resulted in a high stability. The second, because of its simplicity and reproducibility, the large scale production could be developed.

3.1.1 Percent protein entrapment

The percent protein entrapment of protein extract from *Pasteurella multocida* in liposome was showed in Table 11. The percent protein entrapment of liposome 8A was 63.69 and 55.93 percent at 0 and 3 month after storage at 4°C, that of freshly prepared liposome 2T35 lot A and 2T35 lot B was 59.69 and 57.62 percent.

3.1.2 Particle size analysis

Table 12 showed the particle size of liposome containing protein extract from *Pasteurella multocida* .The particle size was measured by Mastersizer, based on the principle of laser ensemble light scattering.

The protein concentration of freshly prepared liposome of *P. multocida* 8A, and 2T35 lot A at 2 mg/ml showed the diameter of 30.12 and 23.34 μm respectively. The liposome 2T35 lot B prepared from 1 mg/ml showed the median diameter of 12.88 μm. The result indicated that protein concentration incorporated in liposome affected the size of liposome. The higher protein concentration, the larger vesicle size was the size of liposome 8A at 0,1,2 and 3 months was 30.12, 30.68, 30.61, and 30.99 μm, respectively. The result indicated that the liposome still contained the same size during the time tested: 3 months for liposome 8A.

The differences in particle size distribution of freshly prepared liposome 8A, and 2T35 and that of three-month storage at 4°C of liposome 8A were showed in figure 9 and 10,respectively. The particle size distribution of liposome and the cumulative undersize frequency curve of liposome 8A, and 2T35 were also showed in appendix I and II.

3.1.3 Determination of dichloromethane residue in liposome

The determination of dichloromethane (DCM) remaining in liposome during the primary emulsification step was done by using gas chromatography. The column was packed with 5% SE-30 and maintained at 50°C, the injection part and the detection part were maintained at 180°C. The peak response showed up within 0.9 minute after sample injection.

The peak area of standard dichloromethane of liposome were showed in table 13. The average value of dichloromethane calculated from calibration standard curve

as shown in Figure 11 was 4.57 $\pm$ 0.03 ppm/ml. Dichloromethane is toxigenic halogented hydrocarbon and the maximum permissible concentration is not more than 5×10^4 ppm/ml in blood.

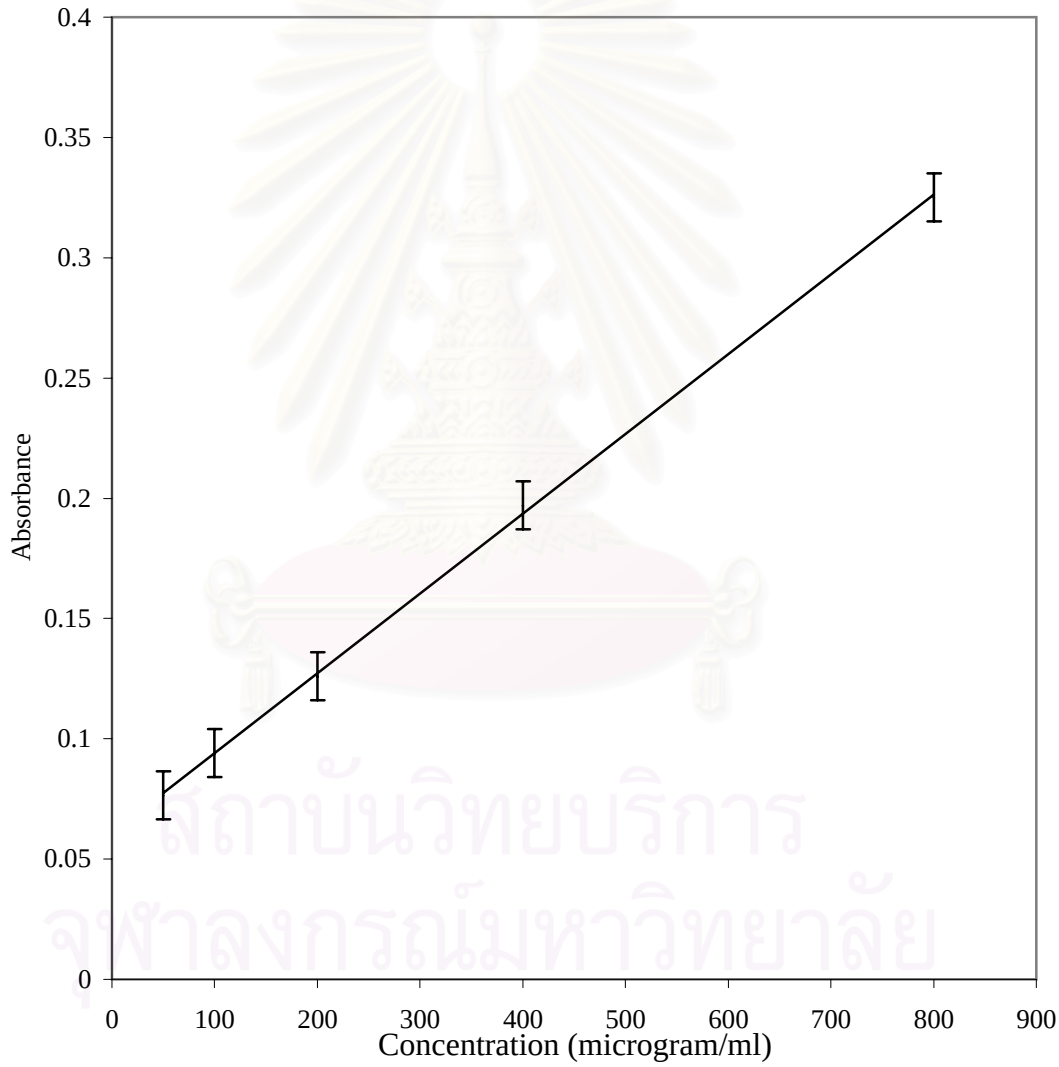
Table 13 Peak area of standard dichloromethane and dichloromethane in liposome

Type of sample	Peak area
Standard 5 ppm.	2550
Standard 10 ppm.	3653
Standard 25 ppm.	4704
Standard 50 ppm.	10440
Standard 75 ppm.	15310
Diluent (Hexane)	1066
Solution A No. 1	1906
Solution A No. 2	1658
Solution B No. 1	2790
Solution B No. 2	2579
Diluent (PBS pH 7.4)	1172

3.1.4 Determination of potassium thiocyanate in protein extract

Given orally at dose of 0.1 to 0.5 percent to female rats during pregnancy and for two weeks after birth, potassium thiocyanate caused growth inhibition in the new borns . Potassium thiocyanate produced goiter in new born lamb when the lamps were exposed orally during pregnancy. Thiocyanate reached the fetus in mice and rats when given intravenously to pregnant animal. Because thiocyanate is a cyanide metabolite, chronic potassium thiocyanate poisoning would be expected to resemble chronic dietary cyanide exposure, with interference with thyroid function and degeneration of the optic nerve and spinal cord. Potassium thiocyanate produces acute toxicity over exposure; fatalities have occurred following ingestion. For this reason the residue of potassium thiocyanate remaining in the protein extract was determined by titration method. The average concentration of potassium thiocyanate detected was 0.0035 $\pm$ 0.0002 %W/V which was non- toxic for animal use (123).

Figure 8 Calibration curve of standard protein in PBS 7.4 at 750 nm
(Assay by Lowry method)



$y = 0.0003x + 0.0608$

$R^2 = 0.9996$

Table11 Percent protein entrapment of antigen in liposome by double emulsion technique with 1:1 molar ratio of lecithin to cholesterol .

Type of antigen in liposome	Month	Entrapping efficiency
Liposome8A	0	63.69
	1	60.49
	2	59.23
	3	55.93
Liposome 2T35 lot A	0	59.69
Liposome 2T35 lot B	0	57.62

Table12 Particle size of liposome by double emulsion technique with 1:1 molar ratio of lecithin to cholesterol

Type of antigen in liposome	Month	Size average (um)
Liposome8A	0	30.12
	1	30.68
	2	30.61
	3	30.99
Liposome 2T35 lot A	0	23.34
Liposome 2T35 lot B	0	12.88

Figure 9 The differences in particle size distribution of freshly prepared liposome 8A, and 2T35

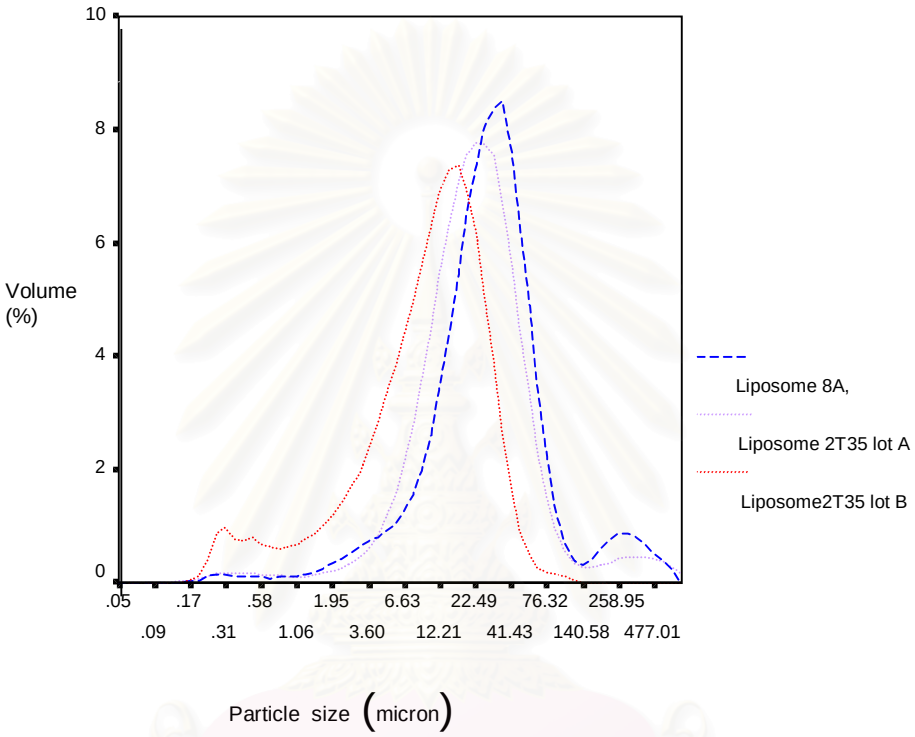


Figure 10 The differences in particle size distribution of three month storage of liposome 8A.

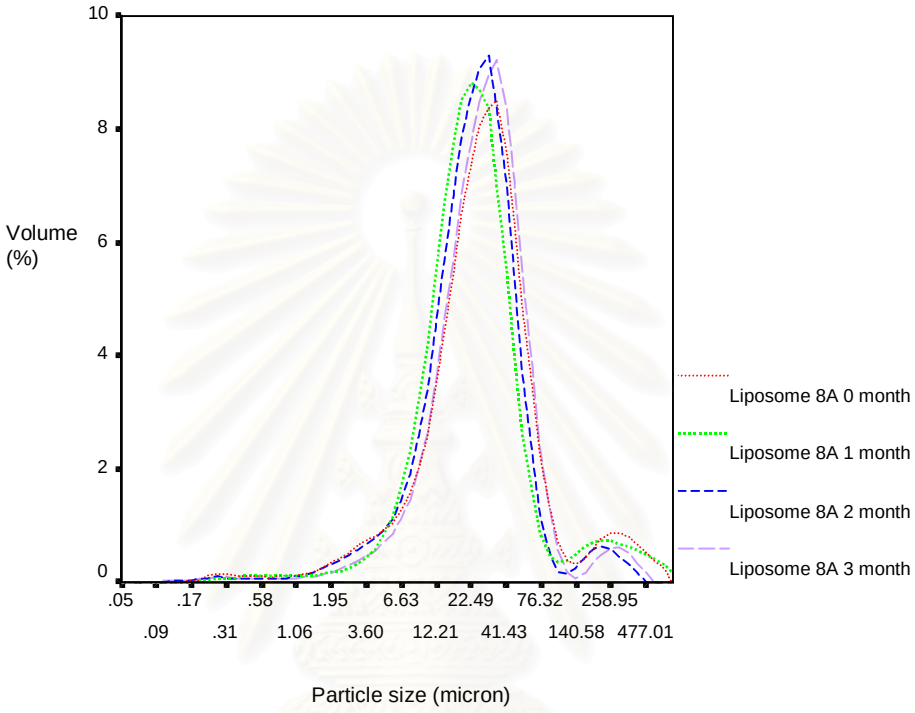
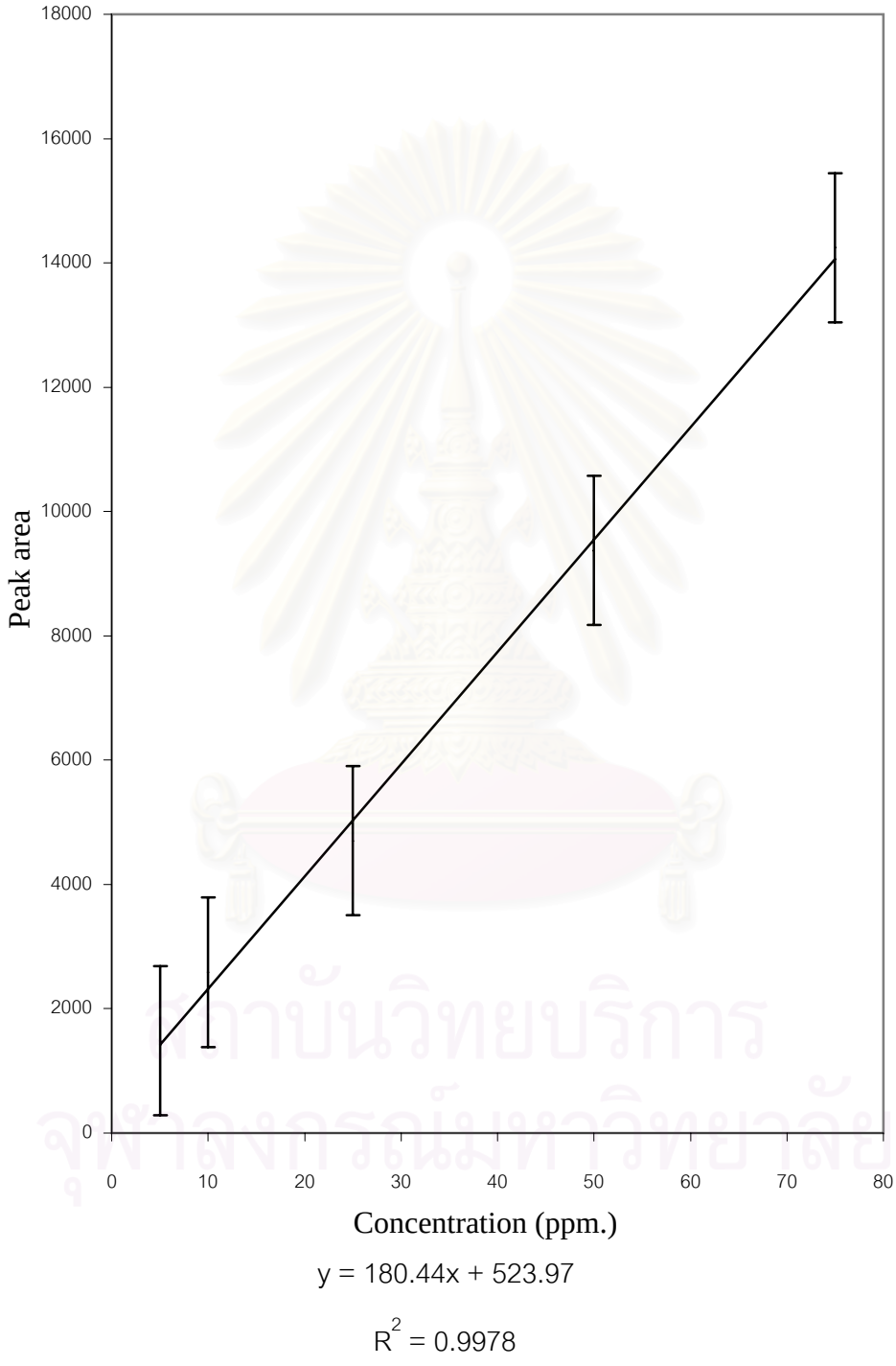


Figure11 Calibration curve of dichloromethane



4. Immunization and determination of immune response

Antibody titer of immune sera from rabbits immunized with KSCN 8A, KSCN 2T35,alum 8A, alum 2T35, liposome 8A, liposome 2T35 lot A, and liposome 2T35 lot B were determined by ELISA. The absorbances of serial dilution of rabbit immune sera reacted with homologous KSCN antigen extract as coating antigen (1 μg/well) in microtiter plate were demonstrated in Table 14,15 and Figure 14,15. All of immune sera containing specific antibodies gave high antibody titer as shown in Figure 12,13. Sera were collected in period of 3 months after vaccination . The difference in antibody response between group interaction was analyzed by ANOVA (Appendix III).

The antibody response of rabbits against KSCN 8A, alum 8A, liposome 8A (Figure 14) detected on day 70, 42 and 63 after the first immunization reached the highest titers of 8,400, 31,000, 72,000, respectively. The antibody responses induced by KSCN 8A on day 49 compared to alum 8A and to liposome 8A were different ($P< 0.05$). The KSCN 8A compared to liposome 8A and alum 8A compared to liposome 8A on day 63, 84 and 91 induced the different antibody response ($P< 0.05$).

It can be seen that antibody titer of antisera against potassium thiocyanate extract 2T35 ,alum 2T35 , liposome 2T35 lot A ,and liposome 2T35 lot B (Figure 15) detected on day 35, 56, 63, and 49 after the first immunization reached the highest titers of 8,800, 39,000, 64,000, 79,000, respectively. The antibody titers induced by liposome 2T35 of different sizes were not significantly different. The responses to KSCN 2T35, alum2T35, liposome 2T35 lot A, and liposome 2T35 lot B were significantly different on day 49. The antibody titer response was different between KSCN 2T35 and liposome 2T35 lot B on day 56 and between KSCN 2T35, alum 2T35 and liposome 2T35 lot A on day 63 ($P < 0.05$). The difference in antibody response liposome 2T35 lot A. and between liposome 2T35 lot A and liposome 2T35 lot B was indicated on day 91, respectively ($P < 0.05$).

In the period of 3 months of immune sera collection, it was indicated that liposome 8A induced the highest antibody titer compared to that of alum 8A and KSCN 8A. Antibody response induced by alum 8A was also higher than that of KSCN 8A. Similarly, the immune response of rabbits immunized with liposome 2T35

elicited the highest value and the antibody titer to alum 2T35 was also higher than that of KSCN 2T35.

The comparison of antibody response of potassium thiocyanate extract 8A and 2T35 was displayed in Figure 16. Both antigen extracts elicited high antibody titer and the antibody responses showed similar pattern. There was no response of antibody to diluent. Figure 17 illustrated the comparison of antibody response to alum protein antigen. The highest antibody titer induced by alum 8A and alum 2T35 was showed on day 42 and 56. As shown in Figure 18, the comparison of antibody response to liposome protein antigen was demonstrated. The higher antibody response was elicited by liposome 2T35 lot B. The response to liposome 8A and liposome 2T35 lot B approximately gave the similar antibody response.

Antibody titers of immune sera from rabbits against KSCN protein extract of heterologous strains were determined by ELISA and the result were demonstrated in Table16- 17. Antibody titers of immune sera reacted to homologous and heterologous whole cell antigens were also determined by ELISA and the results were illustrated in Table 18-21. The antibody titer on day 0,28,63,91 of immune sera specific to homologous strain were concluded in Figure 19.

The result from this study indicated that there were differences in lag times and durations of immune response elicited by different kind of antigen preparations

In addition, the extent of immune response may depend on route of administration, dose and frequency of infection. Further study should be investigated in other animal species especially chicken or ducks.

It was reported that a vaccine prepared from a potassium thiocyanate extract of serotype 3,12,15 *P. multocida* provided protection in rabbits challenged with virulent serotype 3,12,15:D *P. multocida*. The vaccine also elicited serum IgG and nasal mucosal IgA against *P. multocida* (Ringler, D. et al.)(59).

Rabbits intranasal vaccinated with the KSCN extract of a virulent *P. multocida* 3:A developed persisting serum IgG and nasal IgA antibodies, whereas rabbits intramuscular immunized produced persisting serum IgG and transient nasal IgA (Lu, Y.S. et al)(61).

Rabbits vaccinated intranasal or subcutaneous with KSCN extract with toxin had 100 % survival rate upon intranasal challenge, few or no bacteria in the liver and lungs, high serum immune IgG and IgM antibody titer, and significant number of IgG antibody-secreting cells (ASC) in the spleen and tracheobronchial lymph node. Rabbit

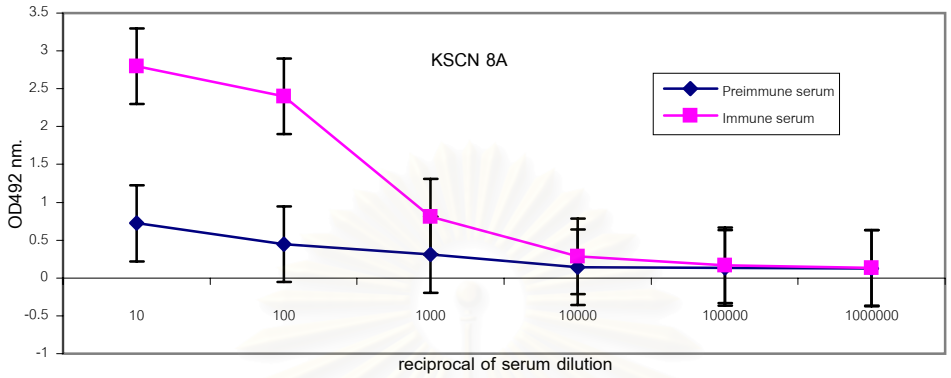
Table 14 Absorbance of rabbit immune sera against KSCN antigen extract 8A determined by ELISA.

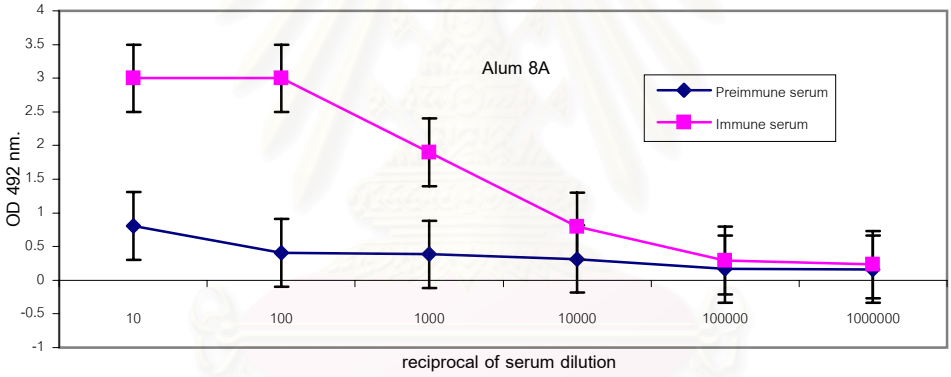
Antigen	Reciprocal of serum dilution	Absorbance of preimmune sera	Absorbance of immune sera
KSCN 8A	10	0.722	2.799
	100	0.444	2.401
	1,000	0.307	0.807
	10,000	0.143	0.283
	100,000	0.136	0.169
	1,000,000	0.129	0.134
Alum 8A	10	0.806	3.000
	100	0.407	3.000
	1,000	0.385	1.900
	10,000	0.316	0.799
	100,000	0.165	0.292
	1,000,000	0.162	0.231
Liposome 8A	10	1.257	3.000
	100	0.573	3.000
	1,000	0.259	3.000
	10,000	0.167	1.578
	100,000	0.146	0.175
	1,000,000	0.137	0.160

Table 15 Absorbance of rabbit immune sera against KSCN antigen extract 2T35 determined by ELISA.

Antigen	Reciprocal of serum dilution	Absorbance of preimmune sera	Absorbance of immune sera
KSCN 2T35	10	1.037	3.000
	100	0.852	3.000
	1,000	0.379	1.199
	10,000	0.155	0.384
	100,000	0.108	0.184
	1,000,000	0.107	0.184
Alum 2T35	10	1.411	3.000
	100	1.229	3.000
	1,000	0.394	1.841
	10,000	0.296	0.655
	100,000	0.202	0.220
	1,000,000	0.189	0.209
Liposome 2T35 lot A	10	0.978	3.000
	100	0.894	3.000
	1,000	0.587	3.000
	10,000	0.207	1.528
	100,000	0.137	0.222
	1,000,000	0.136	0.173
Liposome 2T35 lot B	10	0.717	3.000
	100	0.505	3.000
	1,000	0.359	3.000
	10,000	0.241	1.611
	100,000	0.202	0.312
	1,000,000	0.188	0.188

Figure12 Absorbance of serial dilution of rabbit immune sera against KSCN extract 8A determined by ELISA were compared to those from normal rabbit sera (n = 3). Points indicate mean value, and bars represent standard deviation.





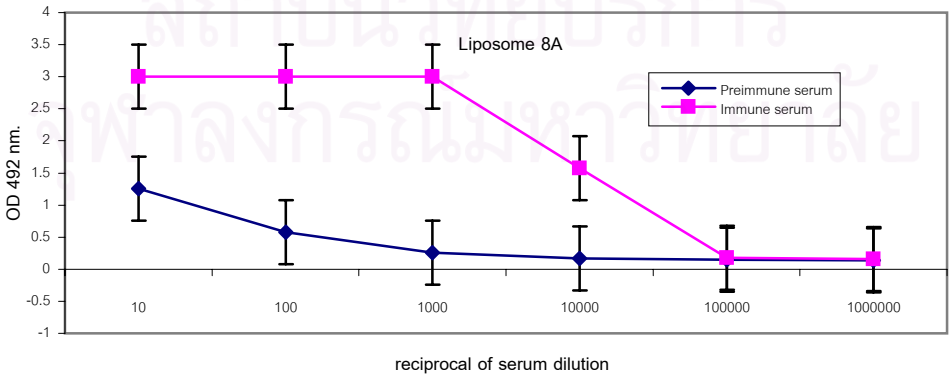
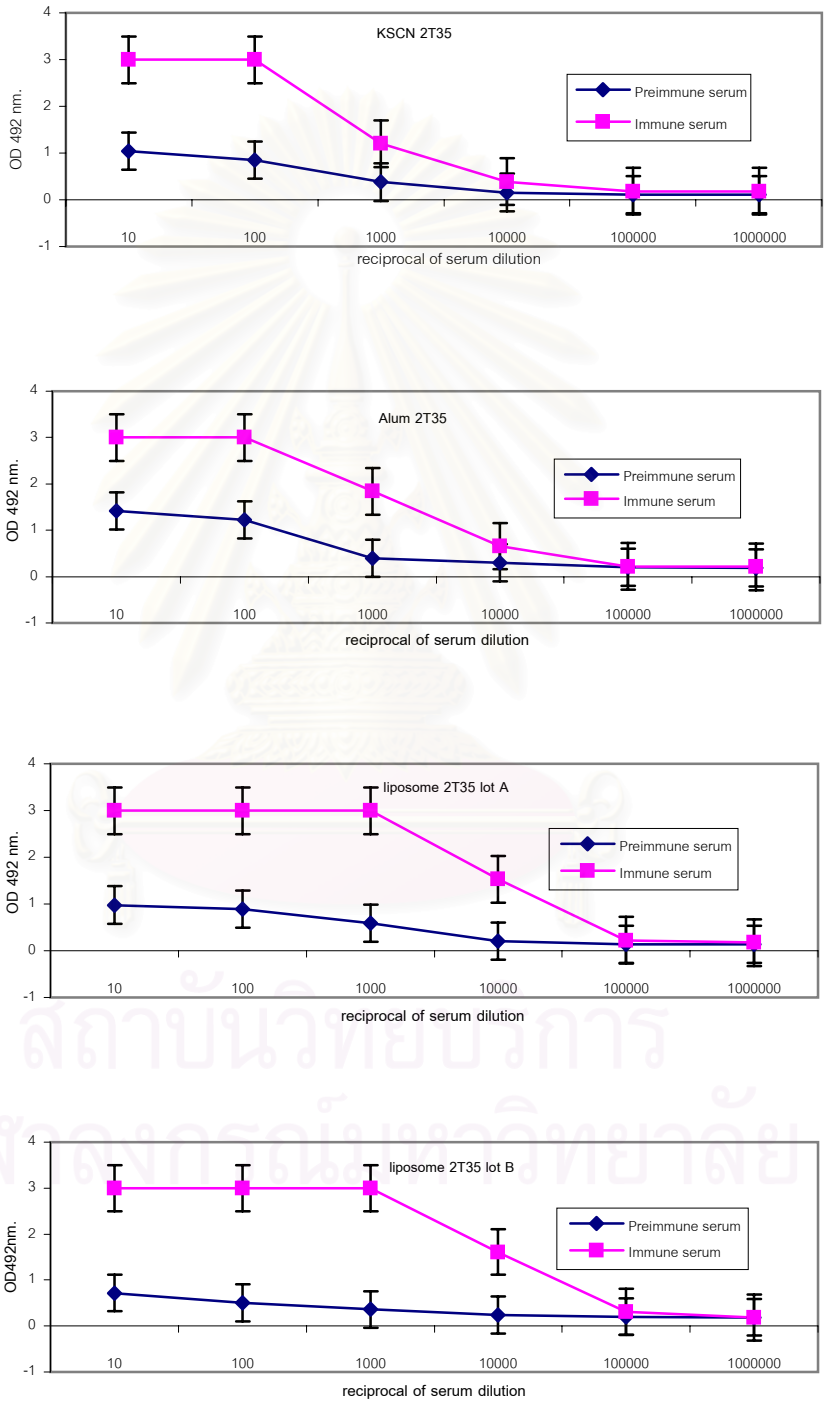


Figure13 Absorbance of serial dilution of rabbit immune sera against KSCN 2T35 determined by ELISA were compared to those from normal rabbit sera (n =3). Points indicate mean values, and bars represent standard deviation.



Antibody titer				
Day	KSCN 8A	Alum 8A	Liposome 8A	PBS
0	50	110	330	360
7	850	380	6400	450
14	2600	5900	32000	490
21	3400	6700	64000	470
28	3700	14000	23000	360
35	5600	25000	37000	540
42	6200	31000	53000	
49	7400	19000	67000	
56	7800	17000	53000	
63	7900	7100	72000	
70	8400	19000	68000	
77	7600	8900	50000	
84	6900	9800	30000	
91	6500	4900	38000	

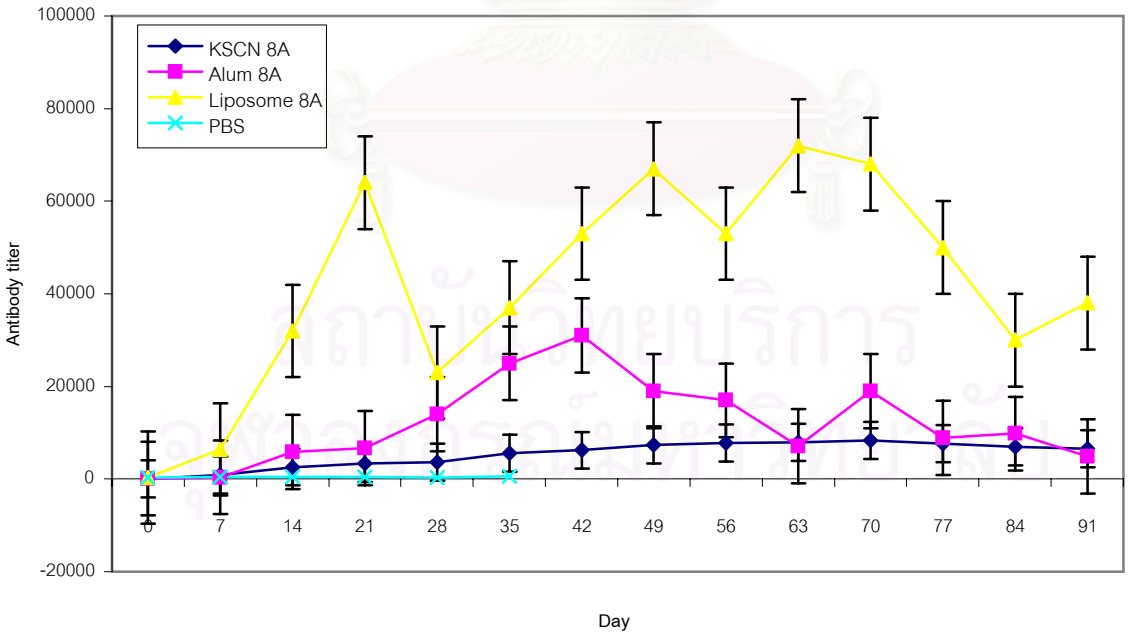


Figure14 Antibody titer determined by ELISA of antisera from rabbits immunized subcutaneously on day 0 and day 7 with KSCN8A, alum8A, liposome8A, and PBS.

Antibody titer					
Day	KSCN 2T35	Alum 2T35	Liposome2T35 lot A	Liposome 2T35 lot B	PBS
0	910	200	400	130	180
7	2400	790	20000	680	370
14	3100	2300	19000	4100	380
21	6600	9100	23500	5700	260
28	8100	9100	6600	39000	410
35	8800	1100	8200	46000	280
42	8400	8100	35000	53000	
49	7000	5800	36000	79000	
56	4900	39000	46000	65000	
63	4100	13000	64000	45000	
70	4500	18000	59000	26000	
77	4800	8500	14500	15000	
84	7400	5600	21300	5100	
91	3000	5800	16700	2300	

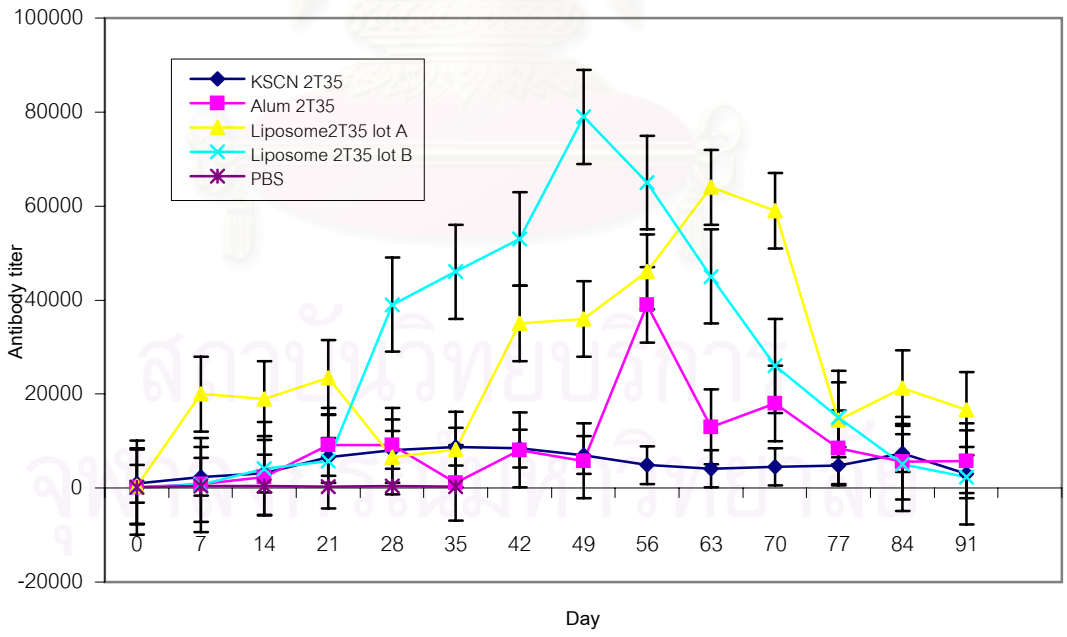


Figure 15Antibody titer determined by ELISA of antisera from rabbits immunized subcutaneously on day 0 and day 7 with KSCN 2T35, alum 2T35, liposome 2T35 lot A, liposome 2T35 lot B and PBS.

Antibody titer(a)				
Day	KSCN 8A	KSCN 2T35	PBS(2T35)	PBS(8A)
0	50	910	180	360
7	850	2400	370	450
14	2600	3100	380	490
21	3400	6600	260	470
28	3700	8100	410	360
35	5600	8800	280	540
42	6200	8400		
49	7400	7000		
56	7800	4900		
63	7900	4100		
70	8400	4500		
77	7600	4800		
84	6900	7400		
91	6500	3000		

a:antiserum was titrated against homologous antigen.

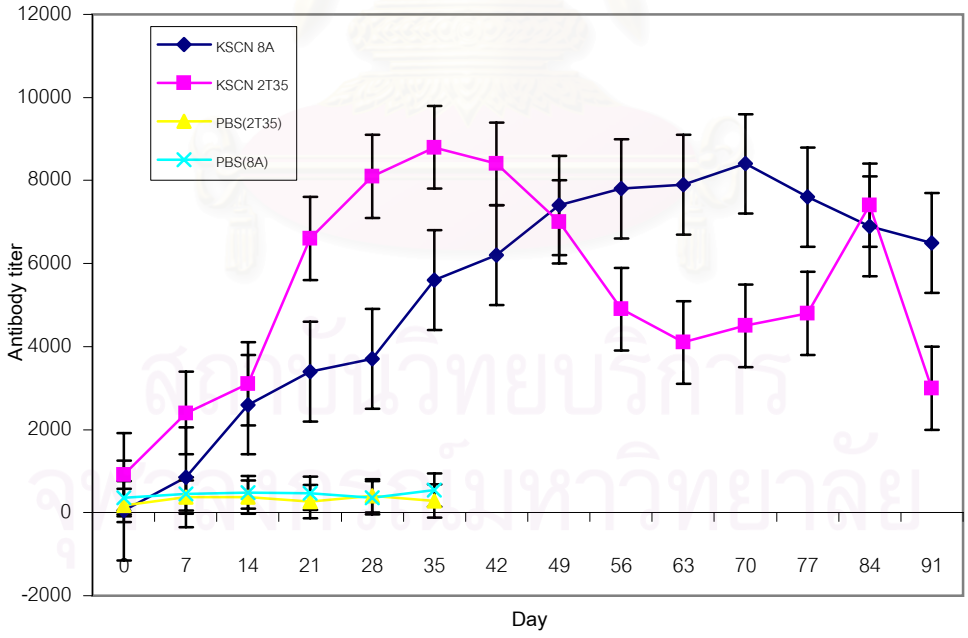


Figure 16 Comparison of antibody titer of immune sera against antigen extract of KSCN 8A, and KSCN 2T35.

Antibody titer(a)		
Day	Alum 8A	Alum 2T35
0	110	200
7	380	790
14	5900	2300
21	6700	9100
28	14000	9100
35	25000	1100
42	31000	8100
49	19000	5800
56	17000	39000
63	7100	13000
70	19000	18000
77	8900	8500
84	9800	5600
91	4900	5800

a : antiserum was titrated against homologous antigen.

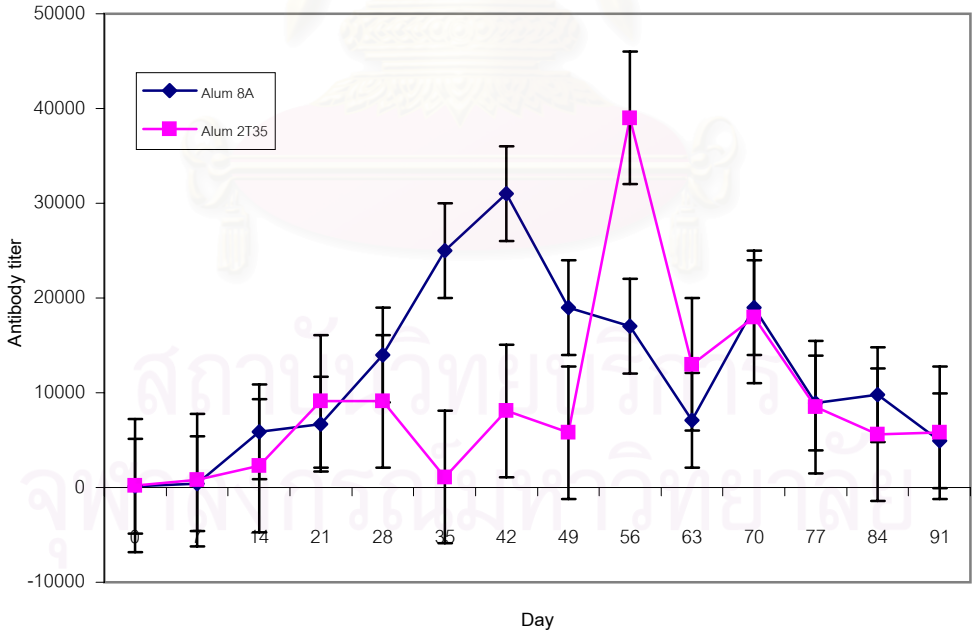


Figure17 Comparison of antibody titer of immune sera against alum 2T35,and alum8A.

Antibody titer(a)			
Day	Liposome 8A	Liposome 2T35 lot A	Liposome 2T35 lot B
0	330	400	130
7	6400	20000	680
14	32000	19000	4100
21	64000	23500	5700
28	23000	6600	39000
35	37000	8200	46000
42	53000	35000	53000
49	67000	36000	79000
56	53000	46000	65000
63	72000	64000	45000
70	68000	59000	26000
77	50000	14500	15000
84	30000	21300	5100
91	38000	16700	2300

a: antisera was titrate of antibody titer against homologous antigen

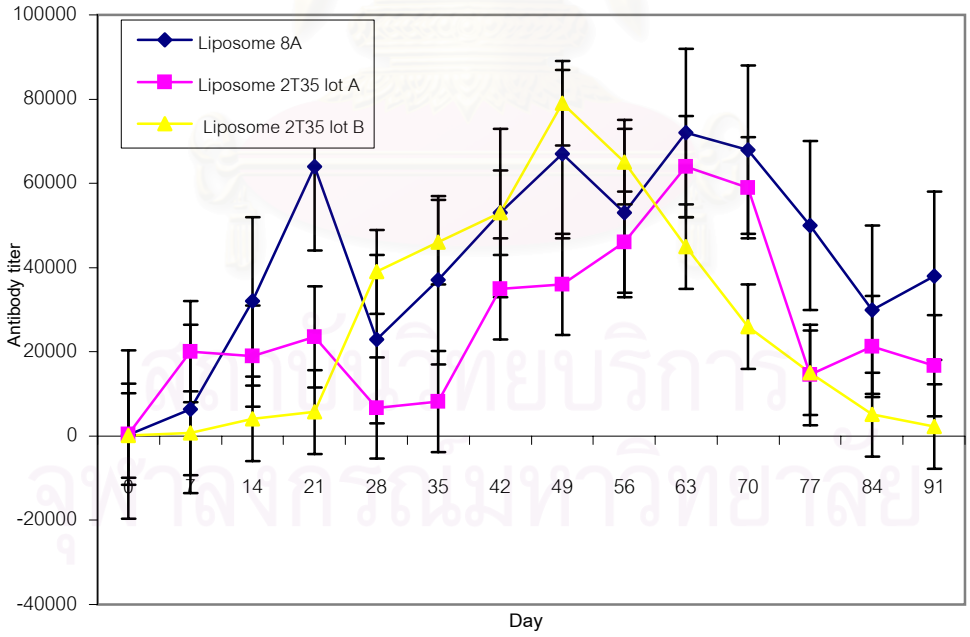


Figure 18 Comparison of antibody titer of immune sera against liposome 8A, liposome 2T35 lotA, and liposome 2T35 lot B.

Table 16 Antibody titers of immune sera from rabbits immunized with KSCN 8A against KSCN 2T35 determined by ELISA

Day	Antibody titer		
	KSCN 8A	Alum 8A	Liposome 8A
0	60	690	910
28	3600	6800	56400
63	7900	10000	54000
91	6700	14800	40000

Table 17 Antibody titers of immune sera from rabbits immunized with KSCN 2T35 against KSCN 8A determined by ELISA.

Day	Antibody titer			
	KSCN 2T35	Alum 2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
0	880	180	120	410
28	9000	8900	39000	6700
63	4500	12000	46000	63000
91	2000	45000	2200	15900

Table 18 Antibody titers of immune sera from rabbits immunized with KSCN 8A against whole cell 8A determined by ELISA.

Day	Antibody titer		
	KSCN 8A	Alum 8A	Liposome 8A
0	57	110	340
28	4900	5600	22000
63	7500	7100	72000
91	5700	5600	39000

Table 19 Antibody titers of immune sera from rabbits immunized with KSCN 2T35 against whole cell 2T35 determined by ELISA.

Day	Antibody titer			
	KSCN 2T35	Alum 2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
0	920	650	910	1100
28	4400	5600	59000	12000
63	9700	9200	46000	69000
91	4800	8800	12000	7900

Table 20 Antibody titers of immune sera from rabbits immunized with KSCN 8A against whole cell 2T35 determined by ELISA.

Day	Antibody titer		
	KSCN 8A	Alum 8A	Liposome 8A
0	65	120	290
28	5000	6100	24000
63	7600	7000	70000
91	5800	6000	37000

Table 21 Antibody titers of immune sera from rabbits immunize with KSCN 2T35 against whole cell 8A by ELISA.

Day	Antibody titer			
	KSCN 2T35	Alum 2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
0	860	620	850	990
28	5700	5400	61000	12700
63	9500	8900	47000	69700
91	4700	8300	11000	7600

Day	Antibody titer						
	KSCN 8A	Alum 8A	Liposome 8A	KSCN 2T35	Alum 2T35	Liposome 2T35lotA	Liposome 2T35lotB
0	50	110	330	910	200	400	130
28	3700	14000	23000	8100	9100	6600	39000
63	7900	7100	72000	4100	13000	64000	45000
91	6500	4900	38000	3000	5800	16700	2300

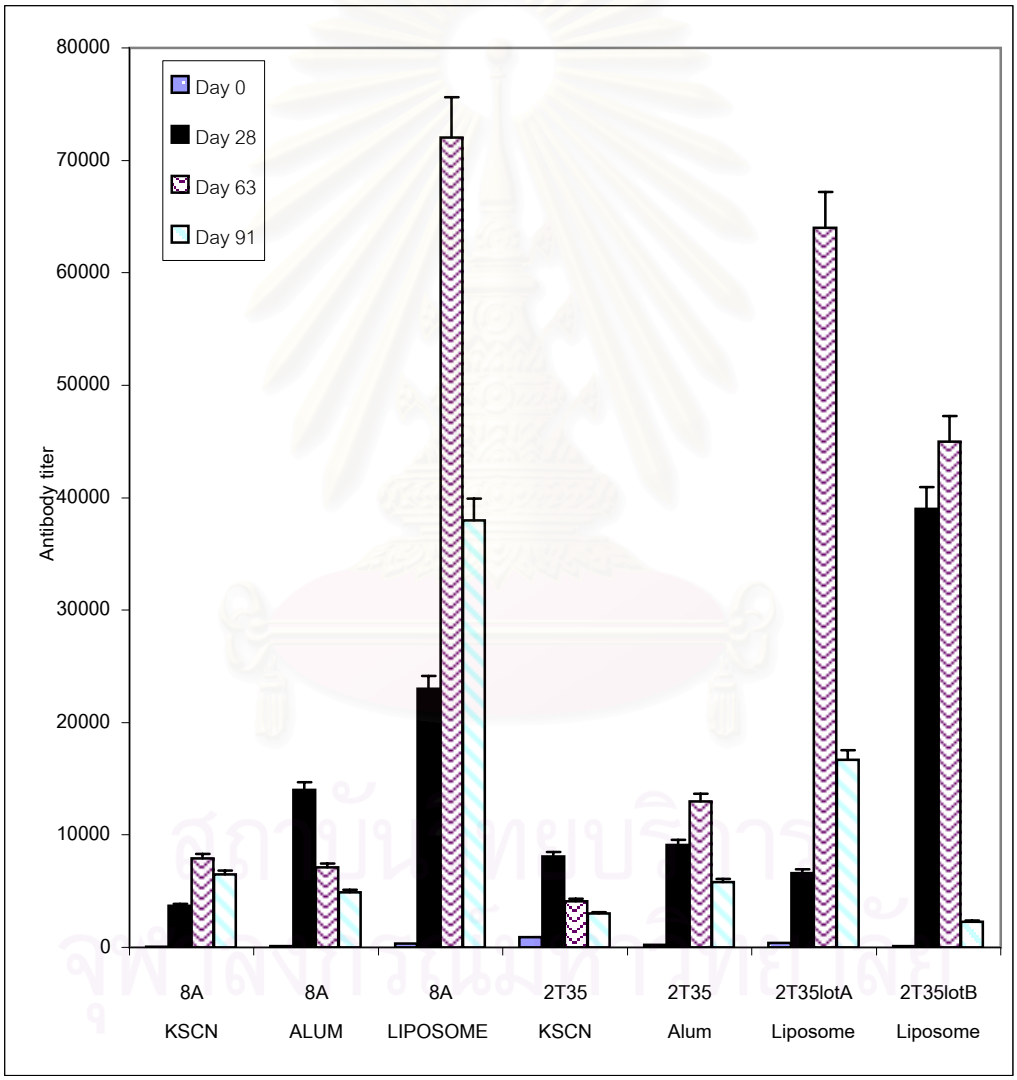


Figure19 Comparison of antibody titer of immune sera against KSCN 8A, alum8A, liposome8A, KSCN2T35, alum2T35, liposome2T35lot A, liposome 2T35 lot B determined by ELISA on day 0, 28, 63, 91

vaccinated intranasal had significant nasal and bronchoalveolar IgA antibody level (Jarvinen L.Z. et al.)(62).

It was also demonstrated that hyperimmune serum containing a high titer of IgG antibody directed to the KSCN extract of *P. multocida* protected against homologous challenge. It was evident that serum IgG antibody to *P. multocida* played a role in protection (Lu, Y.S. et al.)(57).

The coadministration of cholera toxin with potassium thiocynate extract of *P. multocida* leaded to enhanced anti-KSCN antibody activity and increased protection of rabbits against infection with *P. multocida*. The marked increase of antibody in serum (IgG) and nasal lavage (IgA) was detected (Suckow, M.A. et al.)(61).

5. Determination of bactericidal activity of immune sera

Bactericidal activities against homologous strain of immune sera from rabbits were showed in Table 22,23. The comparisons between bactericidal activities and antibody titers of immune sera against KSCN antigen extract 8A and whole cell 8A determined by ELISA were exhibited in Figure 20-22. The antibody titers reached maximum on day 63. The antibody response to KSCN 8A, and to whole cell 8A, and bactericidal activity showed similar pattern. The immune sera obtained from rabbits immunized with liposome 8A elicited higher bactericidal capacity than immune sera from rabbits immunized with alum 8A and KSCN 8A ($P < 0.05$).

The statistic corelation between bactericidal activities and antibody titers against KSCN extract and against whole cell in Figure 20-22. Antibody titers of anti-KSCN 8A against KSCN 8A and whole cell 8A were correlated with bactericidal activity. The bactericidal capacity of anti-alum 8A was correlated with antibody titer against KSCN extract but bactericidal activity of anti-liposome 8A was correlated with antibody titer against whole cell 8A.

The correlations between bactericidal activities and antibody titers of anti-KSCN 2T35, anti-alum 2T35, anti-liposome 2T35 lot A and lot B sera against KSCN antigen extract 2T35 and against whole cell 2T35 were demonstrated in Figure 23-26. The increase and decrease in antibody titers and bactericidal capacity showed similar pattern. Most of the antibody titers against whole cell 2T35 and bactericidal activities giving maximum value on day 63 were observed. For anti-liposome 2T35 lot B, the

maximum extent of response was showed on day 28. The antibody titer against whole cell of anti-KSCN 2T35, anti-alum 2T35, anti-liposome 2T35 lotA, anti-liposome 2T35 lotB were correlated with bactericidal activities.

Bactericidal activities against heterologous strain of immune sera from rabbits were showed in Table 24, 25. The comparisons between heterologous bactericidal activities and antibody titers of immune sera against KSCN antigen extract 2T35 and whole cell determined by ELISA were showed in Figure 20-26. In Figure 20-22, the antibody titers reached maximum on day 63. The antibody response to KSCN 8A, whole cell 8A, and bactericidal activities showed similar pattern. The immune sera obtained from rabbits immunized with liposome 8A elicited higher bactericidal capacity than immune sera from rabbits immunized with alum 8A and KSCN antigen extract 8A. ($P < 0.05$). From Figure 23-26, the increase and decrease in antibody titers and bactricidal capacity showed similar pattern. Most of the antibody against whole cell 8A and bactericidal activities giving maximum value on day 63 were observed. For anti-liposome 2T35 lot B, the maximum response was showed on day 28.

Hyperimmune rabbit sera against whole cell 8A and 2T35 were included as positive control. The bactericidal capacity of positive control was 79.78 and 81.82 %, respectively. Complement and barbital buffer that included as negative control showed zero percent bactericidal capacity. The bactericidal data reported in this study clearly showed that anti-KSCN sera can kill *P. multocida* under the experimental test conditions. As the bactericidal reaction is important for protection, the finding of killing activity provides some theoretical basis for understanding how antibody might be efficient in animal protection against infection.

The investigation of the bactericidal activities of sera suggested that a cell-surface antigen was associated with this mechanism of immunity, because the bactericidal capacity of sera was closely correlated with whole-cell ELISA titers. Although the normal sera showed no bactericidal activities, the immune sera showed higher degree of killing. It may be very difficult to reach killing as high as 90 or 95 % with the amount of available antibody. In the more general case of disease, the force of this situation will depend on the individual balance between infection and resistance, and whether a 50 to 80 % reduction in the number of organisms, with help from other immunity factors such as phagocytes, will be decisive for recovery, or whether all, or nearly of the organisms must be removed by the effective bactericidal reaction.

Table22 Bactericidal activity of a rabbit immune sera tested with *P.multocida 8A.* (a)

Day	Anti-KSCN 8A	Anti-alum 8A	Anti-liposome 8A
0	6.30±0.01	4.8±0.02	5.40±0.01
28	61.05±0.04	53.49±0.11	66.22±0.06
63	61.74±0.02	59.11±0.09	74.37±0.05
91	53.72±0.07	52.54±0.03	69.53±0.03

a : bactericidal activity was showed as % killing

Table23 Bactericidal activity of a rabbit immune sera tested with *P.multocida2T35.* (a)

Day	Anti-KSCN 2T35	Anti-alum 2T35	Anti-liposome 2T35 lot A	Anti-liposome 2T35 lot B
0	5.50±0.01	0	6.08±0.01	4.70±0.02
28	61.32±0.05	57.57±0.01	73.12±0.73	62.54±0.14
63	67.05±0.02	64.38±0.03	63.46±0.21	69.89±0.59
91	62.29±0.09	59.14±0.02	52.25±0.06	63.01±0.04

a : bactericidal activity was showed as % killing

Table24 Bactericidal activity of a rabbit immune KSCN 8A sera test with *P. multocida 2T35.* (a)

Day	Anti-KSCN 8A	Anti-alum 8A	Anti-liposome 8A
0	0	5.21±0.06	5.25±0.04
28	60.68±0.03	54.72±0.07	67.62±0.21
63	61.78±0.01	60.88±0.05	75.74±0.10
91	52.87±0.05	53.38±0.08	69.29±0.07

a: bactericidal activity was showed as %killing

Table25 Bactericidal activity of a rabbit immune KSCN 2T35 sera test with *P. multocida 8A.* (a)

Day	Anti-KSCN 2T35	Anti-alum 2T35	Anti-liposome 2T35 lot A	Anti-liposome 2T35 lot B
0	0	0	2.81±0.01	3.57±0.01
28	60.72±0.15	57.42±0.17	74.01±0.73	62.47±0.23
63	67.41±0.67	62.93±0.32	63.17±0.09	68.48±0.85
91	61.7±0.16	59.91±0.13	52.77±0.13	62.3±0..45

a: bactericidal activity was showed as % killing

Day	Anti-KSCN 8A against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	630	60	60	65	6.30	0
28	8700	3600	4900	5000	61.05	60.78
63	9300	7900	7500	7600	61.74	61.78
91	6600	6700	5700	5800	53.72	52.87

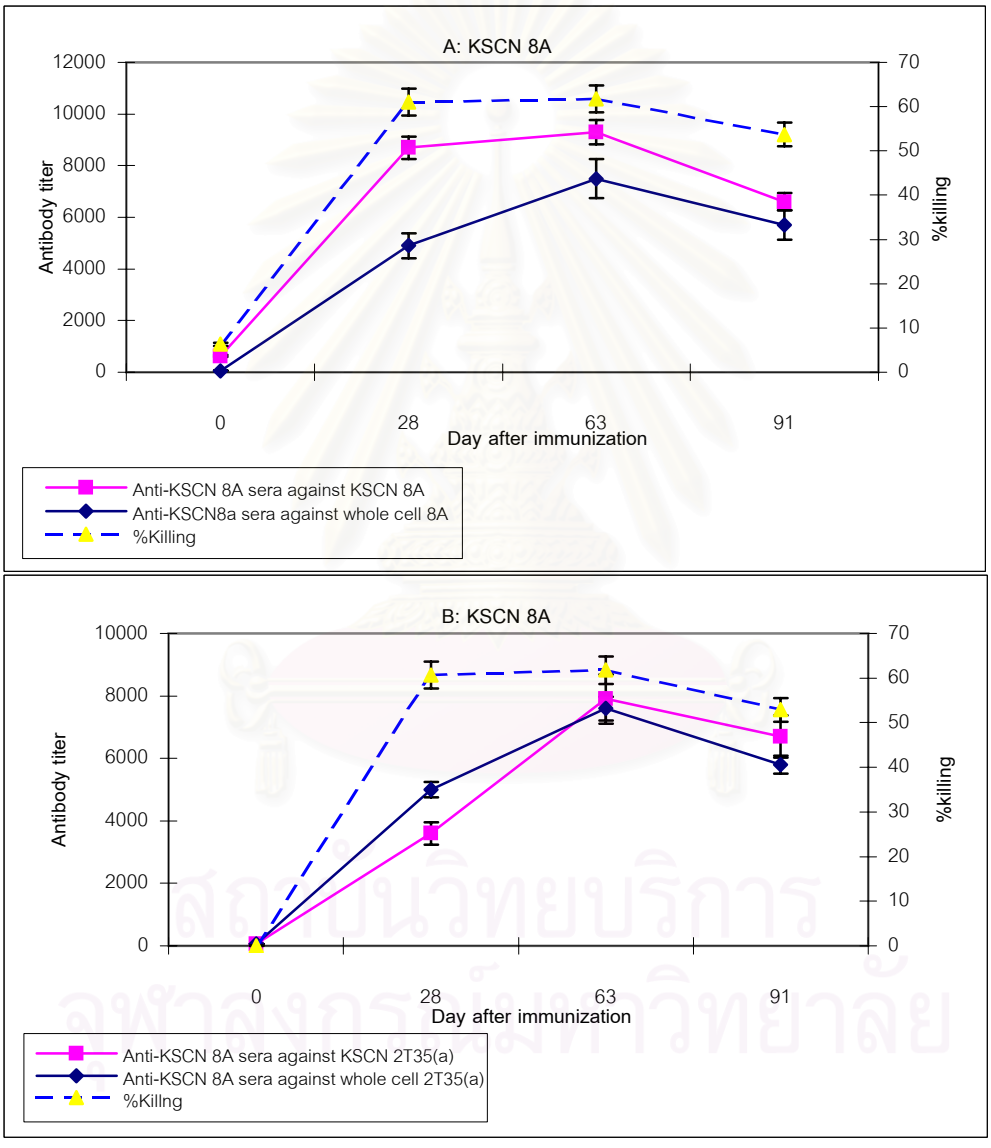


Figure20 Comparison between percent killing of bactericidal activity and antibody titer of anti-KSCN 8A sera against homologous KSCN and whole cell antigen (A) and heterologous KSCN and whole cell antigen(B)

Day	Anti-alum 8A against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	680	690	110	120	4.80	5.21
28	6500	6800	5600	6100	53.49	54.72
63	9900	10000	7100	7000	59.11	60.88
91	14000	14800	5600	6000	52.54	50.00

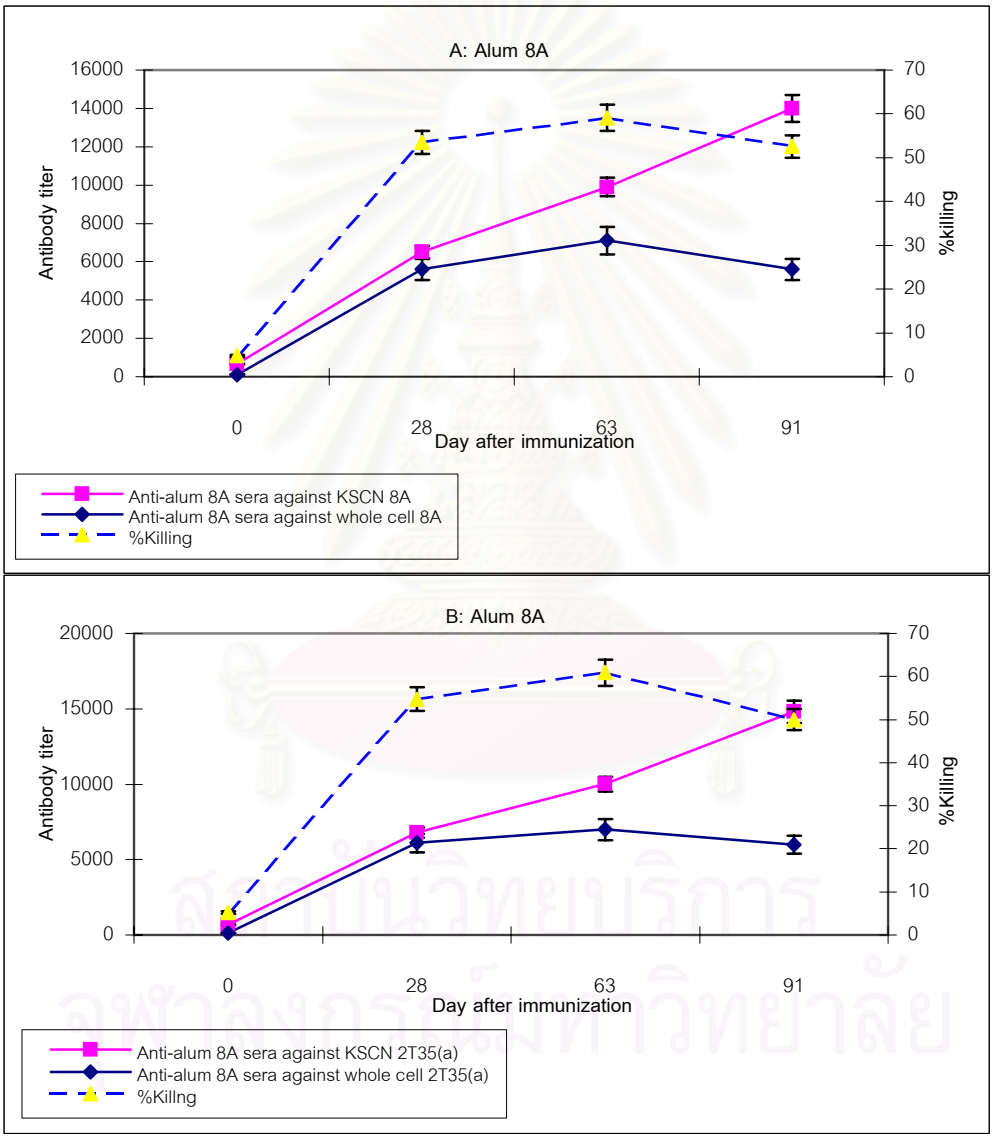


Figure21 Comparison between percent killing of bactericidal activity and antibody titer of anti-alum 8A sera against homologous KSCN and whole cell antigen(A) and heterologous KSCN and whole cell antigen(B)

Day	Ant-liposome 8A against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	1300	910	330	290	5.40	5.25
28	56000	56000	22000	24000	66.22	67.62
63	53000	54000	72000	70000	74.37	75.74
91	41000	40000	39000	37000	69.53	69.29

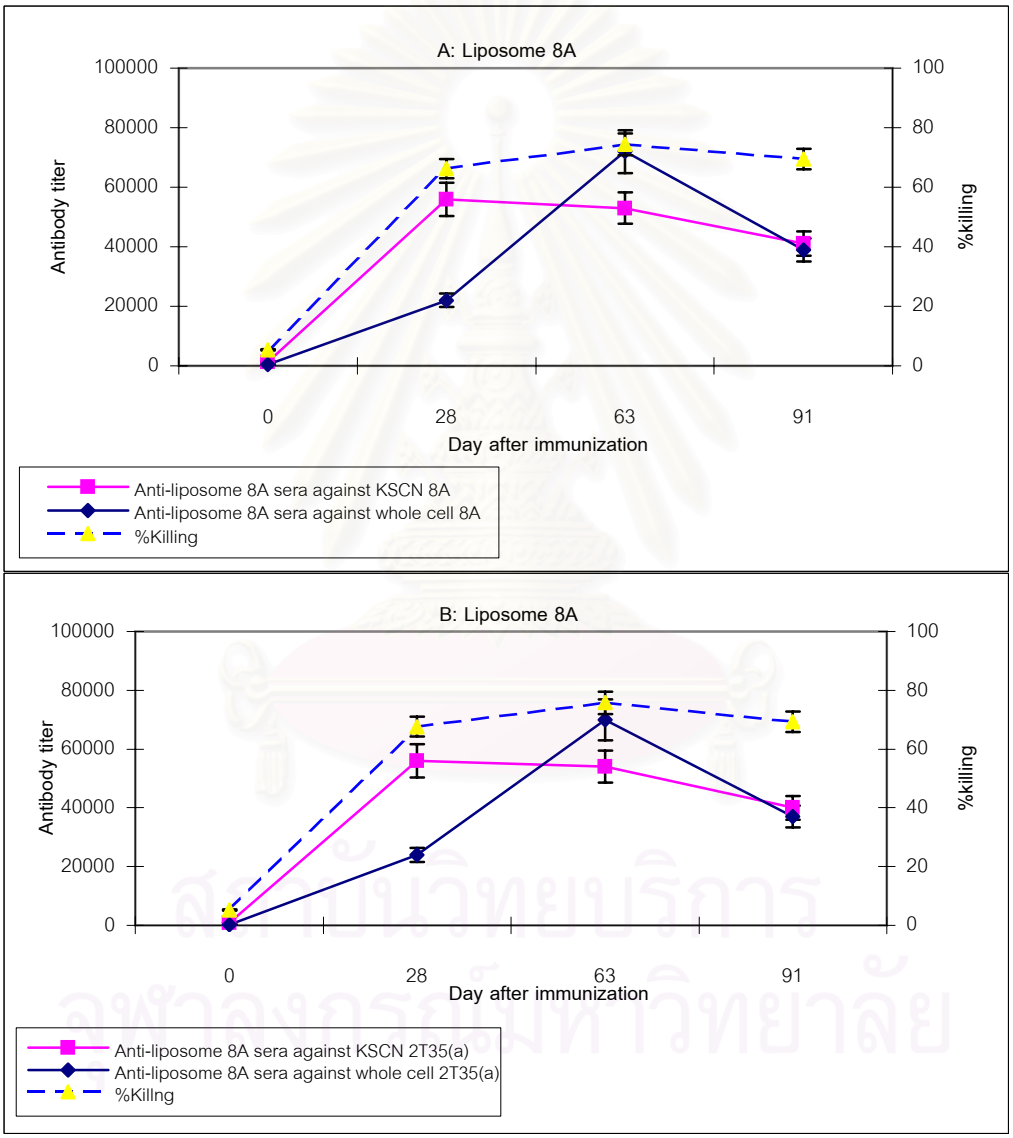


Figure22 Comparison between percent killing of bactericidal activity and antibody titer of anti-liposome 8A sera against homologous KSCN and whole cell antigen (A) and heterologous KSCN and whole cell antigen(B)

Day	Anti-KSCN 2T35 against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	880	910	860	920	0	5.50
28	9000	8100	5700	4400	60.72	61.32
63	4500	4100	9500	9700	67.41	67.05
91	2000	3000	4700	4800	61.70	62.29

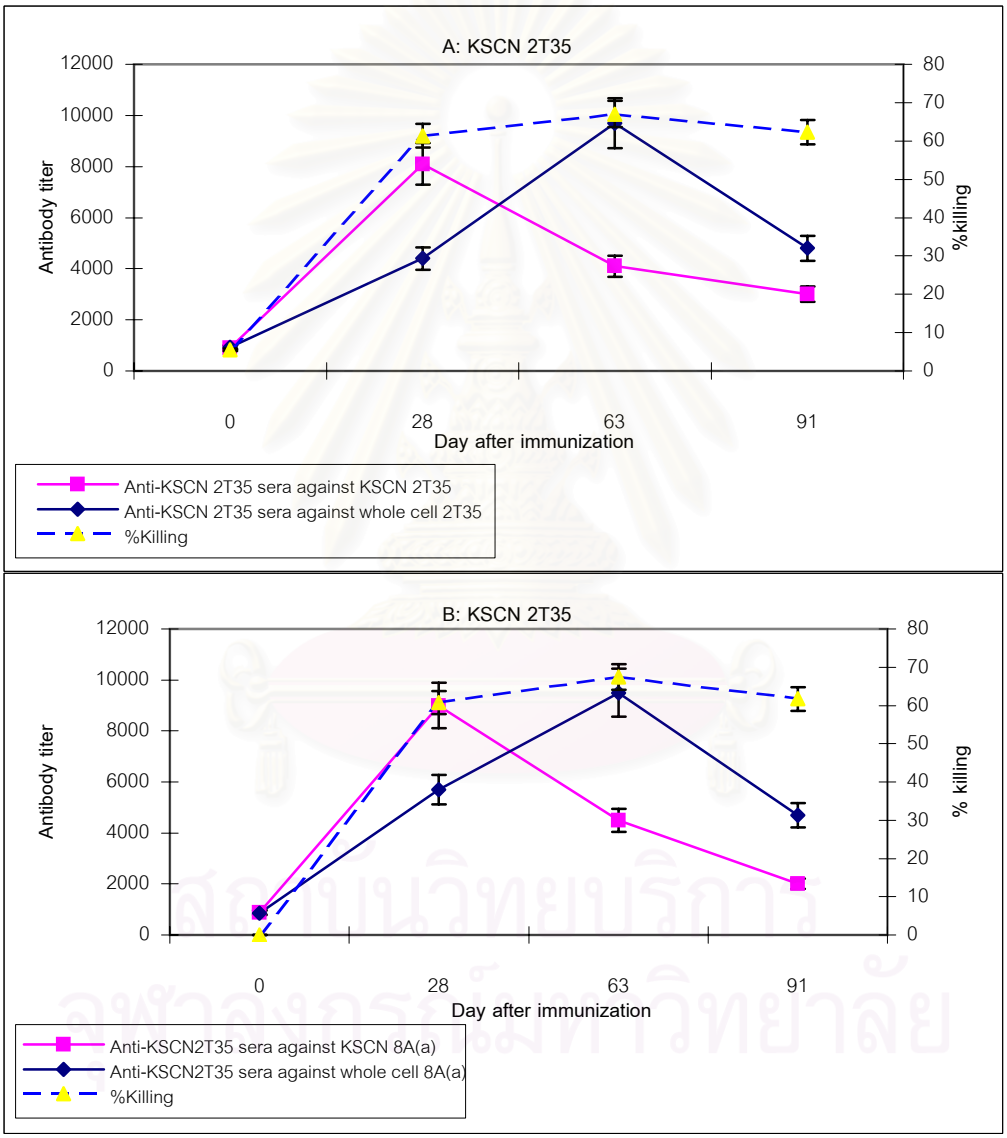


Figure23 Comparison between percent killing of bactericidal activity and antibody titer of anti-KSCN2T35 sera against homologous KSCN and whole cell antigen (A) and heterologous KSCN and whole cell antigen (B)

Day	Anti-alum 2T35 against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	180	200	620	650	0	0
28	8900	9100	5400	5600	57.42	57.57
63	12000	13000	8900	9200	62.93	64.38
91	45000	48000	8300	8800	59.91	59.14

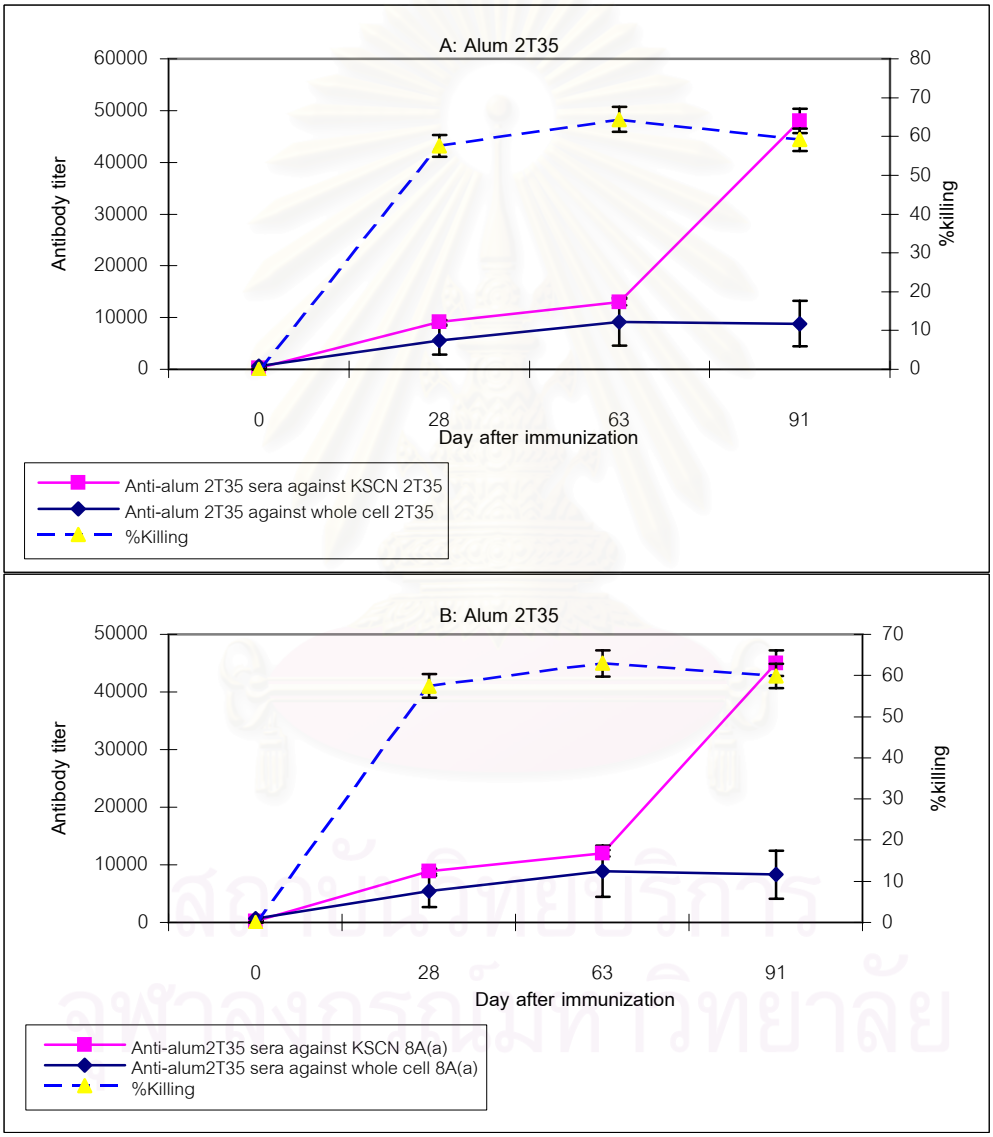


Figure24 Comparison between percent killing of bactericidal activity and antibody titer of anti-alum 2T35 sera against homologous KSCN and whole cell antigen (A) and heterologous KSCN and whole cell antigen (B)

Day	Anti-liposome 2T35 lot A against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	120	130	850	910	2.81	6.08
28	39000	39000	61000	59000	74.01	73.12
63	46000	45000	47000	46000	63.17	63.46
91	2200	2300	11000	12000	52.77	52.25

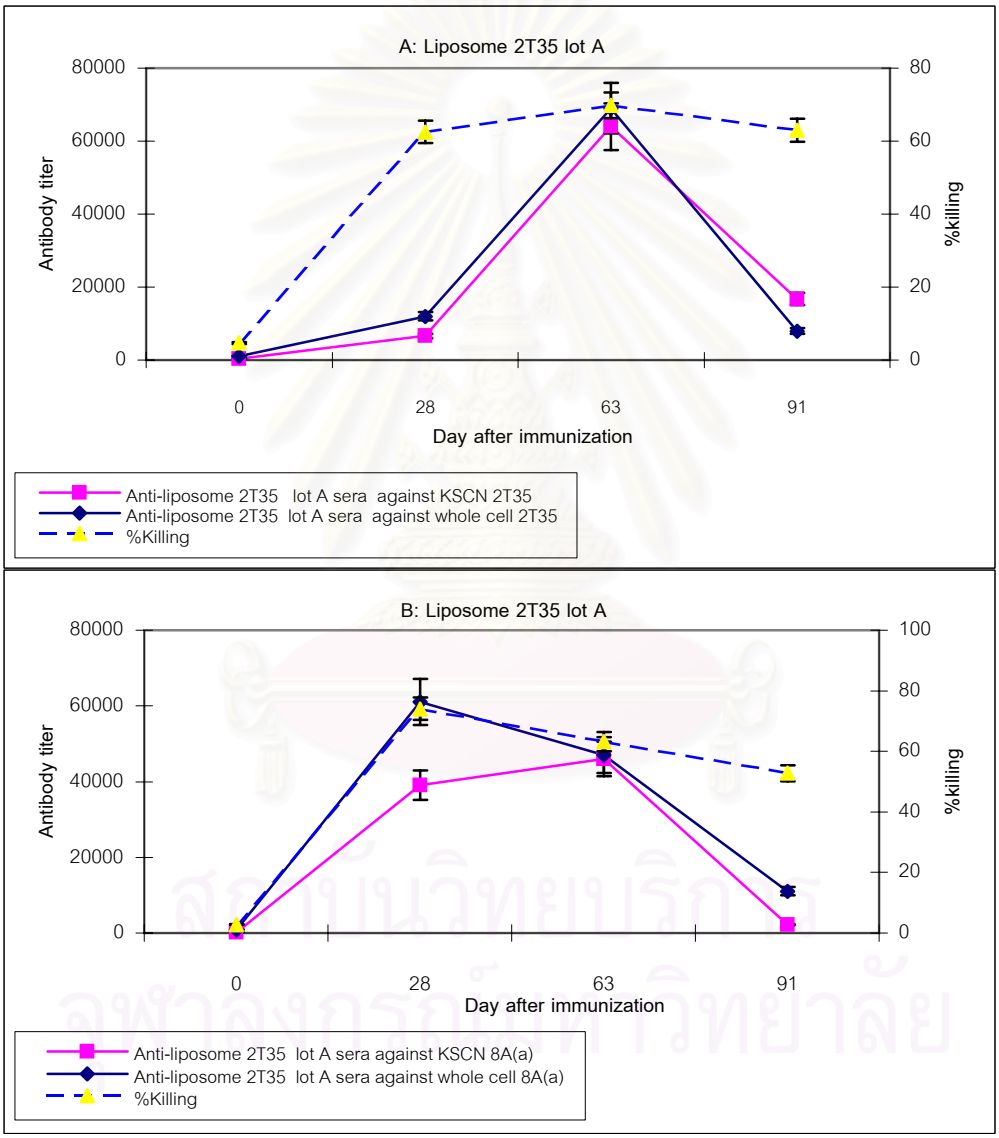


Figure25 Comparison between percent killing of bactericidal activity and antibody titer of anti-liposome 2T35 lot A sera against homologous KSCN and whole cell antigen (A) and heterologous KSCN and whole cell antigen (B)

Day	Anti-liposome 2T35 lot B against				% Killing	
	KSCN 8A	KSCN 2T35	Whole cell 8A	Whole cell 2T35	8A	2T35
0	410	400	990	1100	3.57	4.70
28	6700	6600	12700	12000	62.47	62.54
63	63000	64000	69700	69000	68.48	69.89
91	15900	16700	7600	7900	62.30	63.01

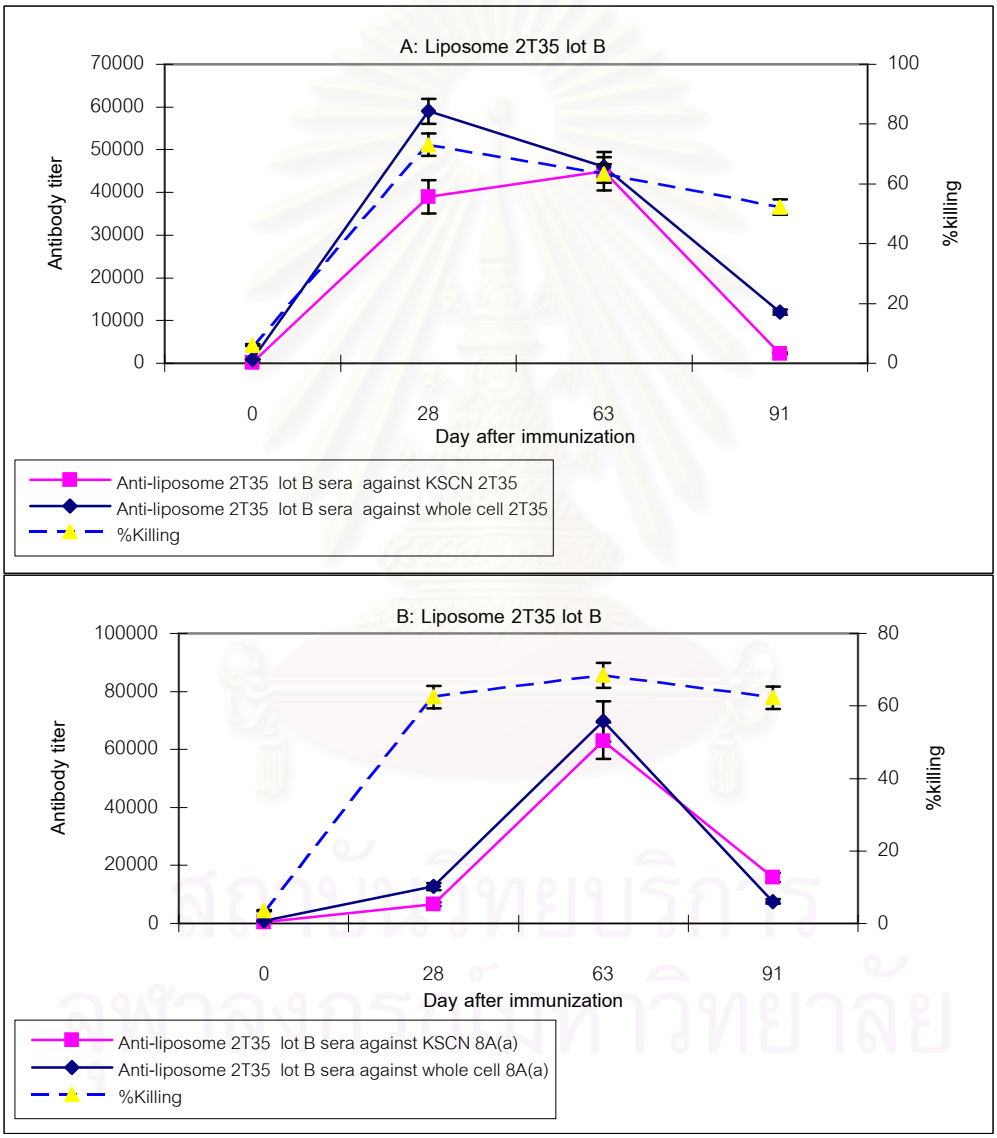


Figure26 Comparison between percent killing of bactericidal activity and antibody titer of anti-liposome 2T35 lot B sera against homologous KSCN and whole cell antigen (A) and heterologous KSCN and whole cell antigen (B)

CHAPTER V

CONCLUSION

The potassium thiocyanate (KSCN) extract of *Pasteurella multocida* 8A and 2T35 was prepared and the average protein concentration was 116 $\pm$ 6.31 µg /mg for *P. multocida* 8A and 82.91 $\pm$ 6.04 µg/mg for *P. multocida* 2T35. Liposome containing protein extracts from *P. multocida* were prepared by double emulsion technique. The percent protein entrapment of liposome 8A, 2T35 lot A (2mg/ml) was 63.69 and 59.69 and that of liposome 2T35 lot B (1 mg protein/ml) was 57.62. After the storage at 4°C for 3 months, the percent protein entrapment of liposome 8A was 60.49, 59.23, and 55.93 % in 1, 2,and 3 month, respectively. The size of freshly prepared liposome 8A, 2T35(A) and 2T35(B) was 30.12, 23.34, and 12.88 µm in diameter. The size of liposome 8A was not different during the storage for 3 months at 4°C. The protein concentration of KSCN extract 2T35 incorporated in liposome did affect the size of liposome: the higher protein concentration the larger vesicle size.

The antibody responses of rabbits elicited from liposome, alum, and potassium thiocyanate extract of *P. multocida* 8A and 2T35 were investigated. The antibody titers of immune sera were determined by ELISA with homologous strain. In the period of 3 months of antisera collection, it was indicated that liposome 8A induced the highest antibody titer compared to that of alum 8A and KSCN 8A. The antibody titer from that of *P.multocida* 2T35 showed similar response pattern. The highest antibody titers exhibited by liposome 8A, liposome 2T35(A) and liposome 2T35 (B) were 72,000, 64,000 and 79,000 on day 63, 63, and 49 after immunization, respectively.

The comparisons between bactericidal activities and antibody titers of anti-KSCN 8A sera against KSCN 8A and whole cell 8A determined by ELISA were studied. Those of anti-KSCN 2T35 sera were performed and the antibody response to whole cell and bactericidal activities showed similar pattern. All antibody titers elicited by KSCN, alum, and liposome, and bactericidal activities showed maximum value on day 63. The maximum killing activities were 69.93, 66.95, and 84.24% for anti-KSCN 8A, anti-alum 8A and anti-liposome 8A, respectively. Anti-KSCN2T35, anti-alum 2T35, anti-liposome 2T35 lot A also showed the maximum killing at day 63

with the value of 75.95, 72.92, and 79.17%, respectively. Bactericidal activity of anti-liposome 2T35 lot B showed the maximum value of 82.82 % on day 28 which was also correlated to antibody titer against whole cell. The bactericidal data reported in this study clearly indicated that anti-KSCN sera can kill *P.multocida* under the experimental test conditions.

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APPENDICES

APPENDIX I

Particle size distribution data
of liposome containing protein extract from *Pasteurella multocida*

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 2T35* (A).

Size low(um)	Frequency%	Size high(um)	Cumulative under%
0.05	0	0.06	0
0.06	0	0.07	0
0.07	0	0.08	0
0.08	0	0.09	0
0.09	0	0.11	0.01
0.11	0	0.13	0.01
0.13	0.01	0.15	0.02
0.15	0.02	0.17	0.03
0.17	0.03	0.20	0.07
0.20	0.07	0.23	0.13
0.23	0.12	0.27	0.26
0.27	0.17	0.31	0.43
0.31	0.17	0.36	0.61
0.36	0.15	0.42	0.76
0.42	0.14	0.49	0.89
0.49	0.13	0.58	1.02
0.58	0.10	0.67	1.12
0.67	0.09	0.78	1.21
0.78	0.08	0.91	1.28
0.91	0.08	1.06	1.36
1.06	0.08	1.24	1.44
1.24	0.09	1.44	1.53
1.44	0.11	1.68	1.64
1.68	0.14	1.95	1.77
1.95	0.17	2.28	1.94
2.28	0.22	2.65	2.16
2.65	0.29	3.09	2.45
3.09	0.38	3.60	2.83
3.60	0.52	4.19	3.35

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 2T35* (A).(cont.)

Size low(um)	Frequency%	Size high(um)	Cumulative under%
4.19	0.72	4.88	4.07
4.88	1.02	5.69	5.09
5.69	1.42	6.63	6.51
6.63	1.97	7.72	8.48
7.72	2.68	9.00	11.16
9.00	3.55	10.48	14.71
10.48	4.59	12.21	19.30
12.21	5.72	14.22	25.02
14.22	6.83	16.57	31.86
16.57	7.79	19.31	39.65
19.31	8.44	22.49	48.09
22.49	8.76	26.20	56.85
26.20	8.86	30.53	65.71
30.53	7.96	35.56	73.67
35.56	6.69	41.43	80.36
41.43	5.24	48.27	85.60
48.27	3.80	56.23	89.40
56.23	2.51	65.51	91.91
65.51	1.48	76.32	93.4
76.32	0.78	88.91	94.18
88.91	0.38	103.58	94.56
103.58	0.23	120.67	94.79
120.67	0.23	140.58	95.02
140.58	0.31	163.77	95.33
163.77	0.39	190.80	95.72
190.80	0.46	222.28	96.19
222.28	0.50	258.95	96.69

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 2T35* (A).(cont.)

Size low(um)	Frequency%	Size high(um)	Cumulative under%
258.95	0.51	301.68	97.20
301.68	0.51	351.46	97.71
351.46	0.49	409.45	98.21
409.45	0.47	477.01	98.68
477.01	0.44	555.71	99.12
555.71	0.39	647.41	99.51
647.41	0.31	754.23	99.82
754.23	0.18	878.67	100

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 2T35(B)* .

Size low(um)	Frequency%	Size high(um)	Cumulative under%
0.05	0	0.06	0
0.06	0	0.07	0
0.07	0	0.08	0
0.08	0	0.09	0
0.09	0	0.11	0
0.11	0	0.13	0
0.13	0	0.15	0
0.15	0	0.17	0
0.17	0.02	0.20	0.02
0.2	0.09	0.23	0.11
0.23	0.38	0.27	0.49
0.27	0.86	0.31	1.35
0.31	0.96	0.36	2.31
0.36	0.76	0.42	3.07
0.42	0.73	0.49	3.81
0.49	0.80	0.58	4.61
0.58	0.66	0.67	5.26
0.67	0.64	0.78	5.91
0.78	0.59	0.91	6.50
0.91	0.61	1.06	7.11
1.06	0.66	1.24	7.78
1.24	0.75	1.44	8.53
1.44	0.87	1.68	9.40
1.68	1.01	1.95	10.41
1.95	1.19	2.28	11.59
2.28	1.38	2.65	12.97
2.65	1.65	3.09	14.62
3.09	1.96	3.60	16.58
3.60	2.36	4.19	18.94

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 2T35(B)* .

Size low(um)	Frequency%	Size high(um)	Cumulative under%
4.19	2.80	4.88	21.74
4.88	3.30	5.69	25.04
5.69	3.82	6.63	28.85
6.63	4.35	7.72	33.21
7.72	4.95	9.00	38.15
9.00	5.60	10.48	43.76
10.48	6.28	12.21	50.04
12.21	6.89	14.22	56.93
14.22	7.30	16.57	64.23
16.57	7.37	19.31	71.6
19.31	6.96	22.49	78.56
22.49	6.13	26.20	84.69
26.2	5.06	30.53	89.75
30.53	3.89	35.56	93.64
35.56	2.63	41.43	96.28
41.43	1.63	48.27	97.90
48.27	0.91	56.23	98.82
56.23	0.47	65.51	99.29
65.51	0.25	76.32	99.54
76.32	0.17	88.91	99.71
88.91	0.14	103.58	99.85
103.58	0.11	120.67	99.96
120.67	0.04	140.58	100
140.58	0	163.77	100
163.77	0	190.8	100
190.8	0	222.28	100
222.28	0	258.95	100
258.95	0	301.68	100

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 2T35(B)* .

Size low(um)	Frequency%	Size high(um)	cumulative under%
301.68	0	351.46	100
351.46	0	409.45	100
409.45	0	477.01	100
477.01	0	555.71	100
555.71	0	647.41	100
647.41	0	754.23	100
754.23	0	878.67	100

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 8A.*

Size low (um)	Frequency%	Size high (um)	Cumulative under%
0.05	0	0.06	0
0.06	0	0.07	0
0.07	0	0.08	0
0.08	0	0.09	0
0.09	0	0.11	0
0.11	0	0.13	0
0.13	0	0.15	0.01
0.15	0.01	0.17	0.02
0.17	0.02	0.20	0.04
0.20	0.04	0.23	0.08
0.23	0.09	0.27	0.17
0.27	0.13	0.31	0.29
0.31	0.13	0.36	0.42
0.36	0.11	0.42	0.53
0.42	0.11	0.49	0.64
0.49	0.11	0.58	0.75
0.58	0.09	0.67	0.84
0.67	0.08	0.78	0.92
0.78	0.09	0.91	1.01
0.91	0.09	1.06	1.10
1.06	0.11	1.24	1.22
1.24	0.15	1.44	1.36
1.44	0.19	1.68	1.55
1.68	0.26	1.95	1.81
1.95	0.34	2.28	2.15
2.28	0.43	2.65	2.58
2.65	0.53	3.09	3.11
3.09	0.63	3.60	3.74
3.60	0.72	4.19	4.46

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 8A.* (cont.)

Size low (um)	Frequency%	Size high (um)	Cumulative under%
4.19	0.81	4.88	5.26
4.88	0.91	5.69	6.17
5.69	1.04	6.63	7.22
6.63	1.24	7.72	8.46
7.72	1.55	9.00	10.01
9.00	1.99	10.48	12.00
10.48	2.61	12.21	14.61
12.21	3.41	14.22	18.03
14.22	4.39	16.57	22.41
16.57	5.46	19.31	27.87
19.31	6.51	22.49	34.39
22.49	7.41	26.20	41.80
26.20	8.04	30.53	49.83
30.53	8.37	35.56	58.20
35.56	8.49	41.43	66.70
41.43	7.62	48.27	74.32
48.27	6.36	56.23	80.68
56.23	4.9	65.51	85.58
65.51	3.45	76.32	89.02
76.32	2.21	88.91	91.23
88.91	1.28	103.58	92.52
103.58	0.68	120.67	93.20
120.67	0.38	140.58	93.57
140.58	0.32	163.77	93.89
163.77	0.42	190.8	94.31
190.8	0.59	222.28	94.91
222.28	0.76	258.95	95.67
258.95	0.86	301.68	96.53
301.68	0.87	351.46	97.40

Particle size distribution data of freshly prepared liposome of *Pasteurella multocida 8A.* (cont)

Size low (um)	Frequency%	Size high (um)	Cumulative under%
351.46	0.80	409.45	98.20
409.45	0.66	477.01	98.86
477.01	0.52	555.71	99.38
555.71	0.38	647.41	99.76
647.41	0.24	754.23	100
754.23	0	878.67	100

Particle size distribution data of liposome of *Pasteurella multocida 8A* after 1 month storage.

Size low(um)	Frequency%	Size high(um)	Cumulative under%
0.05	0	0.06	0
0.06	0	0.07	0
0.07	0	0.08	0
0.08	0	0.09	0
0.09	0	0.11	0.01
0.11	0	0.13	0.01
0.13	0.01	0.15	0.02
0.15	0.01	0.17	0.03
0.17	0.02	0.20	0.05
0.20	0.05	0.23	0.10
0.23	0.08	0.27	0.18
0.27	0.11	0.31	0.28
0.31	0.11	0.36	0.39
0.36	0.10	0.42	0.49
0.42	0.09	0.49	0.58
0.49	0.09	0.58	0.68
0.58	0.09	0.67	0.76
0.67	0.08	0.78	0.85
0.78	0.09	0.91	0.94
0.91	0.11	1.06	1.05
1.06	0.14	1.24	1.18
1.24	0.18	1.44	1.36
1.44	0.23	1.68	1.59
1.68	0.31	1.95	1.90
1.95	0.40	2.28	2.31
2.28	0.50	2.65	2.80
2.65	0.59	3.09	3.39
3.09	0.68	3.60	4.07
3.60	0.75	4.19	4.82

Particle size distribution data of liposome of *Pasteurella multocida 8A* after 1 month storage(cont.).

Size low(um)	Frequency%	Size high(um)	Cumulative under%
4.19	0.81	4.88	5.63
4.88	0.88	5.69	6.51
5.69	0.98	6.63	7.49
6.63	1.16	7.72	8.65
7.72	1.44	9.00	10.09
9.00	1.88	10.48	11.96
10.48	2.5	12.21	14.46
12.21	3.32	14.22	17.77
14.22	4.32	16.57	22.09
16.57	5.43	19.31	27.53
19.31	6.54	22.49	34.06
22.49	7.47	26.20	41.53
26.20	8.11	30.53	49.64
30.53	8.44	35.56	58.08
35.56	8.54	41.43	66.61
41.43	7.58	48.27	74.20
48.27	6.24	56.23	80.44
56.23	4.73	65.51	85.17
65.51	3.26	76.32	88.43
76.32	2.06	88.91	90.49
88.91	1.21	103.58	91.69
103.58	0.72	120.67	92.41
120.67	0.50	140.58	92.92
140.58	0.56	163.77	93.48
163.77	0.76	190.8	94.24
190.80	0.99	222.28	95.23
222.28	1.15	258.95	96.38
258.95	1.16	301.68	97.54
301.68	1.02	351.46	98.56

Particle size distribution data of liposome of *Pasteurella multocida 8A* after 1 month storage(cont.).

Size low(um)	Frequency%	Size high(um)	Cumulative under%
351.46	0.76	409.45	99.32
409.45	0.47	477.01	99.79
477.01	0.21	555.71	100
555.71	0	647.41	100
647.41	0	754.23	100
754.23	0	878.67	100

Particle size distribution data of liposome of*Pasteurella multocida 8A* after 2 month storage.

Size-Low(um)	Frequency%	Size-High(um)	Cumulative under%
0.05	0	0.06	0
0.06	0	0.07	0
0.07	0	0.08	0
0.09	0	0.11	0.01
0.11	0.01	0.13	0.02
0.13	0.01	0.15	0.03
0.15	0.02	0.17	0.05
0.17	0.03	0.20	0.08
0.20	0.05	0.23	0.12
0.23	0.07	0.27	0.19
0.27	0.08	0.31	0.27
0.31	0.09	0.36	0.36
0.36	0.08	0.42	0.44
0.42	0.08	0.49	0.51
0.49	0.07	0.58	0.59
0.58	0.07	0.67	0.65
0.67	0.06	0.78	0.71
0.78	0.06	0.91	0.77
0.91	0.06	1.06	0.83
1.06	0.07	1.24	0.90
1.24	0.09	1.44	0.99
1.44	0.12	1.68	1.11
1.68	0.17	1.95	1.28
1.95	0.23	2.28	1.51
2.28	0.30	2.65	1.81
2.65	0.39	3.09	2.20
3.09	0.47	3.60	2.67
3.60	0.56	4.19	3.23

Particle size distribution data of liposome of*Pasteurella multocida 8A* after 2 month storage(cont.).

Size-low(um)	Frequency%	Size-high(um)	Cumulative under%
4.19	0.65	4.88	3.88
4.88	0.75	5.69	4.63
5.69	0.90	6.63	5.53
6.63	1.11	7.72	6.65
7.72	1.44	9.00	8.09
9.00	1.92	10.48	10.00
10.48	2.58	12.21	12.59
12.21	3.45	14.22	16.04
14.22	4.50	16.57	20.55
16.57	5.67	19.31	26.22
19.33	6.84	22.49	33.06
22.49	7.85	26.20	40.91
26.20	8.60	30.53	49.50
30.53	9.05	35.56	58.55
35.56	9.29	41.43	67.84
41.43	8.40	48.27	76.25
48.27	7.03	56.23	83.23
56.23	5.38	65.51	88.66
65.51	3.71	76.32	92.37
76.32	2.26	88.91	94.63
88.91	1.17	103.58	95.80
103.58	0.49	120	96.29
120.67	0.17	140.58	96.46
140.58	0.13	163.77	96.59
163.77	0.26	190.80	96.84
190.80	0.44	222.28	97.28
222.28	0.59	258.95	97.87
258.95	0.64	301.68	98.51

Particle size distribution data of liposome of*Pasteurella*

multocida 8A after 2 month storage(cont.).

Size-low(um)	Frequency%	Size-high(um)	Cumulative under%
301.68	0.59	651.46	99.10
351.46	0.44	409.45	99.54
409.45	0.30	477.01	99.84
477.01	0.16	555.71	100
555.71	0	647.41	100
647.41	0	754.23	100
754.23	0	878.67	100

Particle size distribution data of liposome of*Pasteurella multocida 8A* after 3 month storage.

Size low(um)	Frequency%	Size high(um)	Cumulative under%
0.05	0	0.06	0
0.06	0	0.07	0.01
0.07	0.01	0.08	0.01
0.08	0.01	0.09	0.02
0.09	0.01	0.11	0.04
0.11	0.02	0.13	0.05
0.13	0.02	0.15	0.08
0.15	0.03	0.17	0.11
0.17	0.04	0.20	0.15
0.20	0.06	0.23	0.21
0.23	0.07	0.27	0.29
0.27	0.09	0.31	0.37
0.31	0.09	0.36	0.46
0.36	0.08	0.42	0.54
0.42	0.08	0.49	0.62
0.49	0.08	0.58	0.70
0.58	0.07	0.67	0.78
0.67	0.07	0.78	0.85
0.78	0.07	0.91	0.92
0.91	0.07	1.06	0.99
1.06	0.08	1.24	1.07
1.24	0.09	1.44	1.16
1.44	0.11	1.68	1.27
1.68	0.14	1.95	1.42
1.95	0.19	2.28	1.60
2.28	0.24	2.65	1.84
2.65	0.30	3.09	2.15
3.09	0.38	3.60	2.53
3.60	0.46	4.19	2.99

Particle size distribution data of liposome of*Pasteurella multocida 8A* after 3 month storage.

Size low(um)	Frequency%	Size high(um)	Cumulative under%
4.19	0.56	4.88	3.55
4.88	0.69	5.69	4.24
5.69	0.86	6.63	5.1
6.63	1.11	7.72	6.21
7.72	1.47	9.00	7.69
9.00	1.99	10.48	9.68
10.48	2.68	12.21	12.35
12.21	3.55	14.22	15.90
14.22	4.59	16.57	20.49
16.57	5.72	19.31	26.2
19.31	6.83	22.49	33.03
22.49	7.80	26.20	40.83
26.2	8.52	30.53	49.35
30.53	8.97	35.56	58.32
35.56	9.24	41.43	67.55
41.43	8.40	48.27	75.95
48.27	7.07	56.23	83.23
56.23	5.47	65.51	88.49
65.51	3.82	76.32	92.31
76.32	2.37	88.91	94.69
88.91	1.26	103.58	95.95
103.58	0.54	120.67	96.49
120.67	0.17	140.58	96.67
140.58	0.08	163.77	96.75
163.77	0.17	190.80	96.92
190.8	0.34	222.28	97.26
222.28	0.50	258.95	97.77
258.95	0.60	301.68	98.37

Particle size distribution data of liposome of*Pasteurella multocida 8A* after 3 month storage.

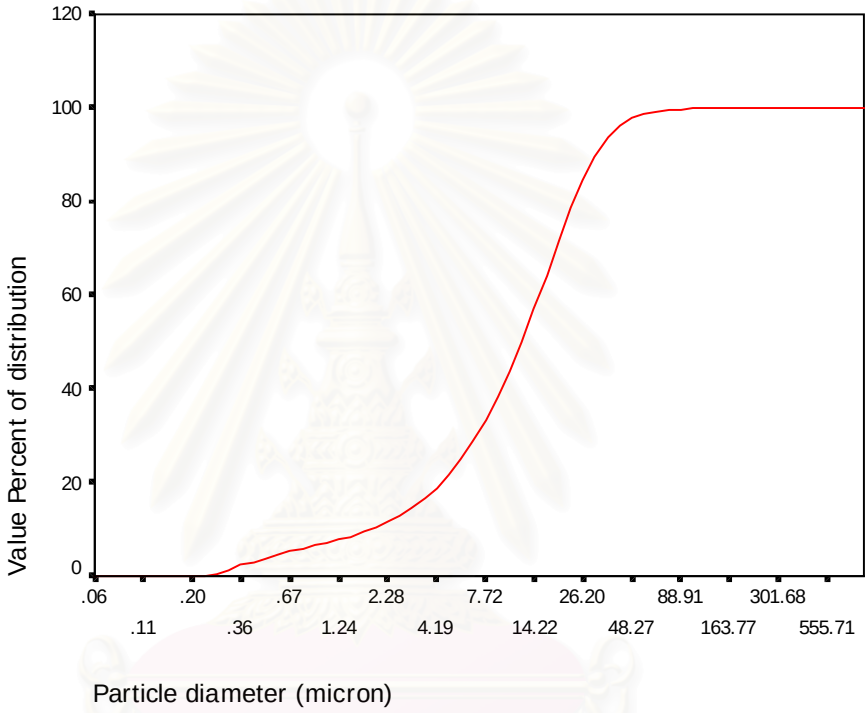
Size low(um)	Frequency%	Size high(um)	Cumulative under%
301.68	0.61	351.46	98.98
351.46	0.51	409.45	99.49
409.45	0.34	477.01	99.83
477.01	0.17	555.71	100
555.71	0	647.41	100
647.41	0	754.41	100
754.23	0	878.67	100

APPENDIX II

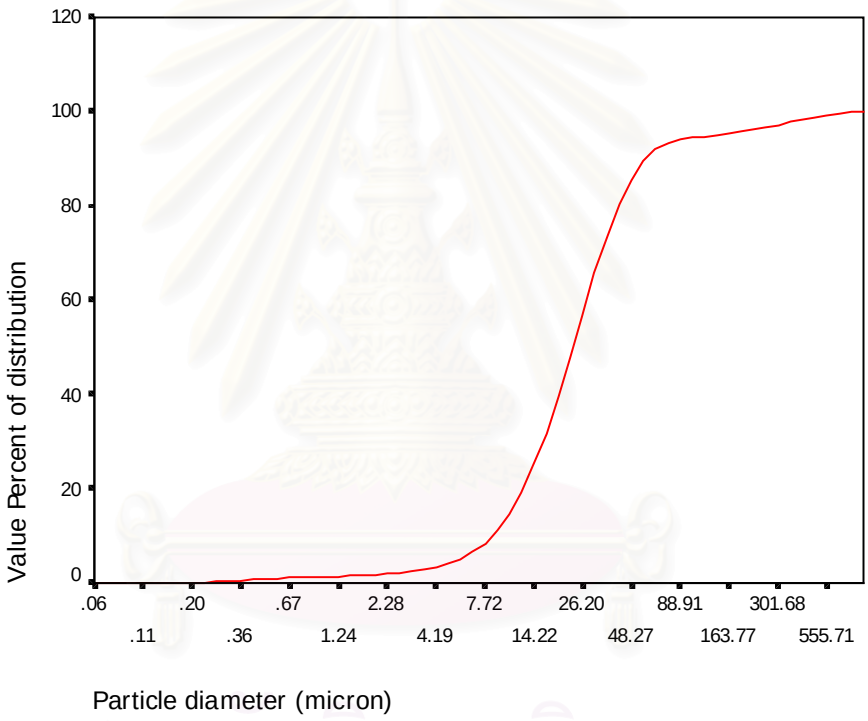
Cumulative undersize frequency curve

of liposome protein extract from *Pasteurella multocida*

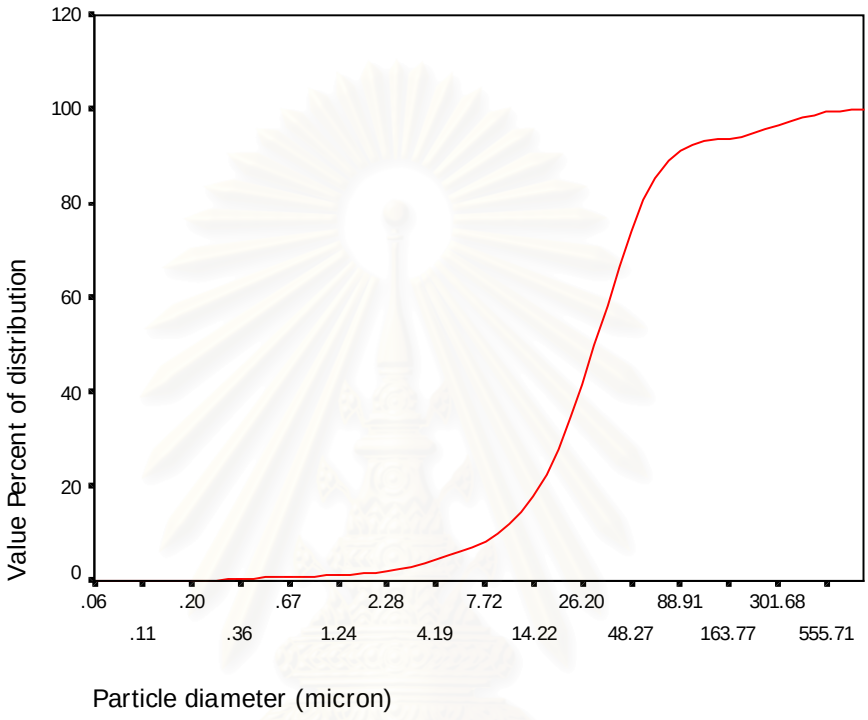
Cumulative undersize frequency curve of liposome of *Pasteurella multocida* 2T35 Lot. B.



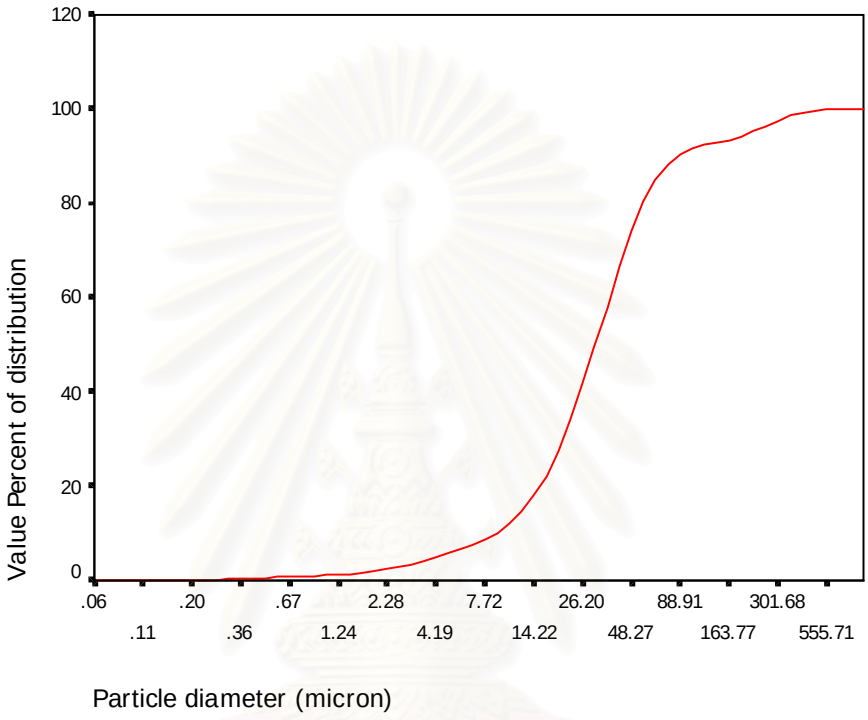
Cumulative undersize frequency curve of liposome of *Pasteurella multocida* 2T35 Lot. A.



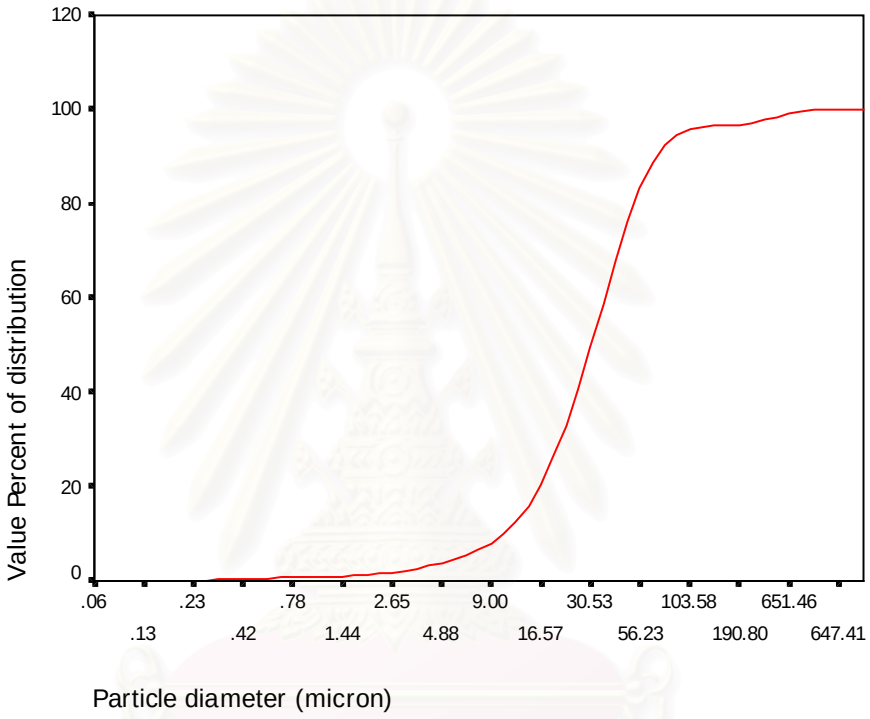
Cumulative undersize frequency curve of liposome of *Pasteurella multocida* 8A.



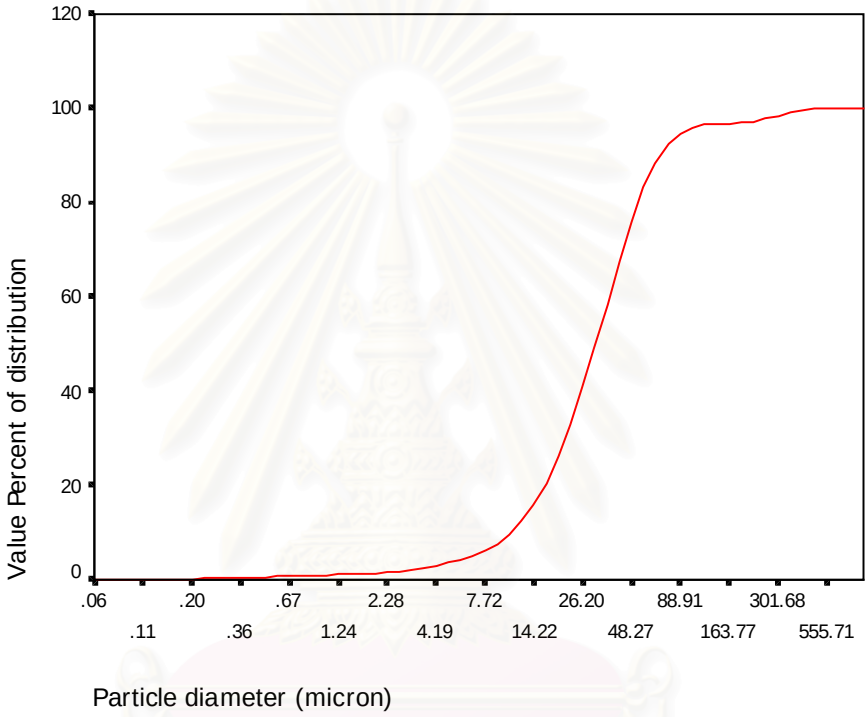
Cumulative undersize frequency curve liposome of *Pasteurella multocida* 8A after one month storage.



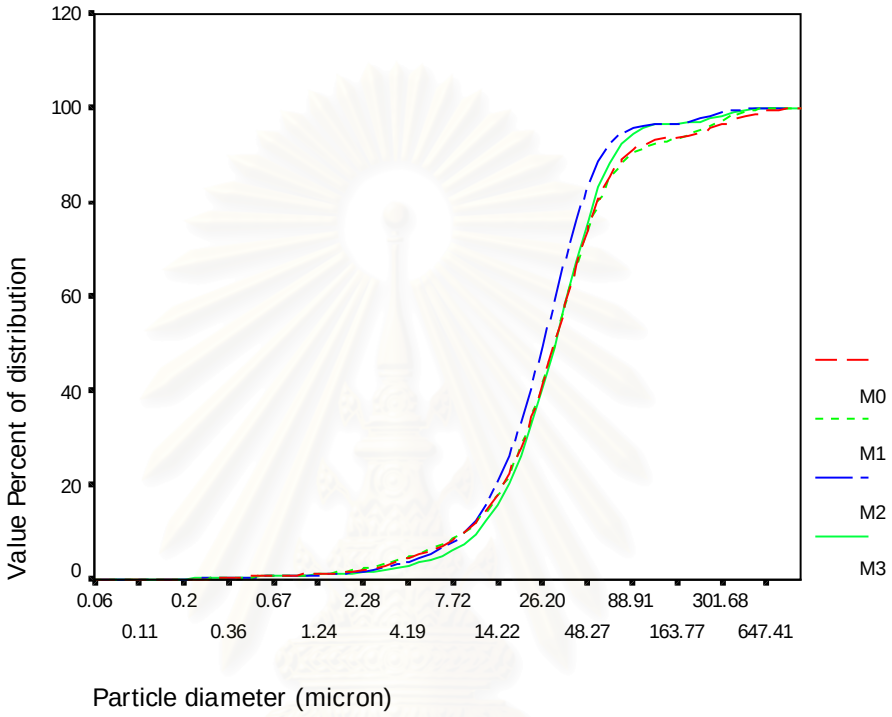
Cumulative undersize frequency curve liposome of *Pasteurella multocida* 8A of 2 months storage.



Cumulative undersize frequency curve liposome of *Pasteurella multocida* 8A of 3 months storage.



Cumulative undersize frequency curve liposome of *Pasteurella multocida* 8A of 1-3 month storage.



APPENDIX III

Statistic significant difference between treament groups on different day

Statistical significant difference between 8:A treament groups on different day, sera were collected in period of 3 months after vaccination.(Analysed by ANOVA)

(Table: A =day 49, B = day 63, C = day 84, D = day 91)

A

	Statistical significant (P) difference		
Treatment group	KSCN 8A	Alum 8A	Liposome 8A
KSCN 8A		0.042	
Alum 8A			
Liposome 8A	0.042		

There were significantly difference in antibody titers between groups on day 49, with a P value < 0.05.

B

	Statistical significant (P) difference		
Treatment group	KSCN 8A	Alum 8A	Liposome 8A
KSCN 8A			0.012
Alum 8A			0.011
Liposome 8A	0.012	0.011	

There were significantly difference in antibody titers between groups on day 63, with a P value < 0.05

C

	Statistical significant (P) difference		
Treatment group	KSCN 8A	Alum 8A	Liposome 8A
KSCN 8A			0.007
Alum 8A			0.019
Liposome 8A	0.007	0.019	

There were significantly difference in antibody titers between groups on day 84, with a P value < 0.05

Statistical significant difference between 8:A treament groups on different day, sera were collected in period of 3 months after vaccination.(Analysed by ANOVA)

(Table: A =day 49, B = day 63, C = day 84, D = day 91)

D

	Statistical significant (P) difference		
Treatment group	KSCN 8A	Alum 8A	Liposome 8A
KSCN 8A			0.001
Alum 8A			0.001
Liposome 8A	0.001	0.001	

There were significantly difference in antibody titers between groups on day 91, with a P value < 0.05

Statistical significant difference between of treament groups on, different day sera were collected in period of 3 months after vaccination.(Analysed by ANOVA)

(Table: A =day 49, B = day 56, C = day 63, D = day 91)

A

Treatment group	Statistical significant (P) difference			
	KSCN 2T35	Alum 2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
KSCN 2T35				0.012
Alum2T35			0.011	0.011
Liposome 2T35 lot A		0.011		
Liposome 2T35 lot B	0.012	0.011		

There were significantly difference in antibody titers between groups on day 49, with a P value < 0.05.

B

Treatment group	Statistical significant (P) difference			
	KSCN 2T35	Alum 2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
KSCN 2T35				0.024
Alum 2T35				
Liposome 2T35 lot A				
Liposome 2T35 lot B	0.024			

There were significantly difference in antibody titers between groups on day 56, with a P value < 0.05.

Statistical significant difference between of treament groups on, different day sera were collected in period of 3 months after vaccination.(Analysed by ANOVA)

(Table: A =day 49, B = day 56, C = day 63, D = day 91)

C

	Statistical significant (P) difference			
Treatment group	KSCN 2T35	ALUM2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
KSCN 2T35			0.019	
ALUM2T35			0.001	
Liposome 2T35 lot A	0.019	0.001		
Liposome 2T35 lot B				

There were significantly difference in antibody titers between groups on day 63, with a P value < 0.05.

D

	Statistical significant (P) difference			
Treatment group	KSCN 2T35	ALUM2T35	Liposome 2T35 lot A	Liposome 2T35 lot B
KSCN 2T35				
ALUM2T35				
Liposome 2T35 lot A				
Liposome 2T35 lot B			0.000	

There were significantly difference in antibody titers between groups on day 63, with a P value < 0.05.

APPENDIX IV

Preparation of media for stock microorganism and reagent solution

Media for stock microorganism

Skim milk

Skim milk	100	g
Distilled water to	1000	ml

Sterile at 121-124°C, 10 minute.

5% blood agar

Tryptic soy agar	40	g
Sheep blood	50	ml
Distilled water	950	ml

Sterile at 121-124 °C, 15 minute before adding sheep blood.

Reagent solution for potassium thiocyanate extraction

1.0 M. Potassium thiocyanate (KSCN MW. = 97.18)

Potassium thiocynate	97.18	g
Distilled water	1000	ml

0.9% Normal saline

Sodium chloride	9	g
Distilled water	1000	ml

Dialysis solution

Tris – hydrochloride	0.01	mol/L
NaCl	0.32	M
NaN_3	0.01	%
Distilled water	1000	ml

Adjust pH to 8.0 before bringing to volume

1.2 M Sodium chloride

Sodium chloride	187.01	g
Distilled water	1000	ml

0.1 M Tris – HCl

Tris – Hydrochloride	15.76	g
Distilled water	1000	ml

0.1% Sodium nitride (NaN_3)

Sodium nitride	1	g
Distilled water	1000	ml

Reagent solution for Alum preparation

1.0M Sodium bicarbonate ($NaHCO_3$)

Sodium bicarbonate	84.01	g
Distilled water	1000	ml

0.2 M Potassium aluminium sulfate ($ALK(SO_4)_3$)

Potassium aluminium sulfate	51.68	g
Distilled water	1000	ml

Reagent solution for ELISA

0.05 M Carbonate bicarbonate buffer pH 9.6 (coating buffer)

Sodium carbonate	0.8	g
Sodium hydrogen carbonate	1.5	g
Distilled water to	500.0	ml

Adjust pH to 9.6 before bring to volume

Citrate – phosphate buffer pH 5.0 (substrate buffer)

Citric acid	9.3	g
Sodium hydrogen phosphate	18.3	g
Thimerosol	0.1	g
Distilled water to	1000	ml

Adjust pH to 5.0 before bringing to volume

Phosphate buffer saline pH 7.4 with Tween 20 (washing buffer)

Sodium chloride	8.0 g
Potassium dihydrogen phosphate	0.2 g
Sodium hydrogen phosphate	2.9 g
Potassium chloride	0.2 g
Thimerosol	0.1 g
Tween 20	0.5 ml

Adjust pH 7.4 before bringing to volume by distilled water to 1000 ml.

1% Bovine serum albumin in PBS –Tween 20 (diluent)

Bovine serum albumin	1.0 g
PBS-T	100.0 ml

3% Bovine serum albumin in PBS-Tween 20 (blocking solution)

Bovine serum albumin	3.0 g
PBS-T	100.0 ml

4N Sulfuric acid

98% Sulfuric acid	54.4 ml
Distilled water to	500.0 ml

Barbital buffer (0.05 mole sodium barbital, 0.01 mole barbital)

Sodium barbital	10.31 g
Barbital	1.84 g
Distilled water	1000 ml

Adjust pH to 8.6 before bringing to volume

VITA

Mr. Anan Watcharadamrongkun was born on June 19, 1973 in Bangkok, Thailand. He graduated with a Bachelor degree of Pharmaceutical Science from Faculty of Pharmaceutical Sciences, Chulalongkorn University in 1997.

Mr. Anan is currently working in the quality control department of research and development in M&H Manufacturing Co. Ltd., (Merck Licence).