VIBRIOCIDAL ANTIBODY AND ANTIBODIES TO *VIBRIO CHOLERAE* LIPOPOLYSACCHARIDE, CELL-BOUND HAEMAGGLUTININ AND TOXIN IN THAI POPULATION

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INTRODUCTION

It was reported that cholera was first introduced into Thailand in 1820, though no statistical records were then available until 1918. Between 1918-1959, there were five outbreaks of cholera in the country. The epidemics had taken place along the trade routes and lines of communication, with the classical biotype as the causative agent. In 1960, the epidemic waned, though sporadic occurrence of cholera was recorded (Morgan *et al.*, 1960). The great nineteenth century cholera pandemic hit Thailand just as the rest of the world. The outbreak in June 1963 was due to El Tor *Vibrio cholerae*. Since then this biotype has replaced the classical vibrios and has established its endemicity firmly in Thailand. One of the factors believed to be responsible for the endemicity of El Tor cholera is the presence of carriers in the communities, in whom the organisms can survive for extended period varying from a few days to a few months (Dizon *et al.*, 1963; 1967; Sinha *et al.*, 1967; Tharavanij *et al.*, 1969). The 1963 outbreak was followed by spread of the infection throughout the country. From 1963 onward, cases were reported every year. However, the outbreaks were not extensive but were rather sporadic in nature. At present, the morbidity and mortality of cholera in Thailand are not as high as in the past, although the transmission of cholera still exist among certain groups of the population with poor environmental sanitation and personal hygiene such as in slums, both in urban and rural areas and people living along the seacoast of the Gulf of Thailand.

Studies on seroepidemiology of cholera would provide information on the background levels of exposure to cholera among the people which will be useful if any new cholera vaccine is to be tested or launched in Thailand.

MATERIALS AND METHODS

Serum samples: Sera were collected from Thai people lived in two different areas. The first area is Samutsakhon province which is about 30 kilometres west of Bangkok. This province is situated on the seacoast and has had more than 25 cases of cholera per 100,000 people per year (Annual Summary; Department of Epidemiology, Ministry of Public Health, Thailand). This area was regarded as "area of high morbidity of cholera (H)" in this study. The second area regarded as "area of low cholera morbidity (L)" is Bangkok metropolis which includes all districts except Phya Thai, Phra Khanong, Ratburana and Bang Khun Thian districts. The L area has had less than 25 cases of cholera per 100,000 individuals per year. The collected serum specimens from each area were divided into 5 different age groups: group 1 (new born-6 months), group 2 (> 6 months-12 months), group 3 (> 1 year-5 years), group 4

(> 5 years-15 years) and group 5 (> 15 years). Samples from newborns were from obstetrics wards of Samutsakhon Provincial and Bangkok Children Hospitals while sera of individuals age more than 7 days to 18 years old were from patients with acute infections, (except diarrhoeas) from the out-patient departments of the two hospitals. Sera of people older than 18 years old were from blood donors. Attempts were made to collect age group matched samples from the two areas during the same season in order to avoid variations due to seasonal prevalence of cholera vibrios (although later experiments showed that there was no seasonal variation in the antibody titres). The sera were heat-inactivated at 56°C for 30 minutes and kept frozen until assay.

V. cholerae antigens: Lipopolysaccharide (LPS) was prepared from *V. cholerae* El Tor, O_{17}SR strain by the phenol-water method of Westphal and Jann (1965). The single extracted LPS was re-extracted until no protein was detected by the conventional biochemical method (Lowry method) and by sodium dodecyl sulphate polyacrylamide gel electrophoresis with Coomassie blue stain. Cholera toxin (CT) was purchased from Sigma Chemical Company. Cell-bound haemagglutinin was produced also from the O_{17}SR strain as follows:- The bacteria were grown in large quantities in Trypticase soy agar for 48 hours at 37°C. The culture was, then, washed 3 times by centrifugation at 10,000 × g at 4°C for 15 min with normal saline solution (NSS). The bacteria were suspended in NSS and subjected to ultraturrax mixer (Janke and Kunkel, West Germany) for 2 hours. The bacterial cells were pelleted by centrifugation as above. The clear supernatant was filtered through a Sephadex G-200 column in Tris-HCl buffer. The fractions with haemagglutinating activity (when mixed with 2.5% sheep red blood cells in NSS) were pooled, dialysed against distilled water at 4°C and lyophilized. The dried material was dissolved in small volume of phosphate buffered saline (PBS), pH 7.2 and passed through a sheep-red cell stroma-Sepharose 4B column. After thorough washing to eliminate the non-adsorbed material, the adsorbed haemagglutinin was eluted out with triple distilled water. The eluate was concentrated by lyophilization. The preparation was called "pure cell-bound haemagglutinin (pure CHA)".

Vibriocidal assay was performed by the technique as described by Rowley (1968). Serial five fold dilutions of each serum were made in peptone saline solution. The viable count of the bacteria were determined before and after incubation at 37°C by the technique of Miles and Misra (1938). Control positive sera were included in each assay. The vibriocidal titre was the highest dilution of the serum which gave at least 50% killing of the vibrios added as compared with the number counted from sample of negative control (peptone saline with vibrios and complement and no antibody).

Control positive sera were sera of cholera patients during the convalescence period. They were obtained from Bamrasnaradura Infectious Diseases Hospital, Ministry of Public Health, Thailand.

Enzyme-linked immunosorbent assay (ELISA) was used to determine levels of immunoglobulin class specific anti-LPS, anti-CHA and anti-CT in the sera. Fifty microlitres of either LPS (50 μg dry weight per ml), CHA (20 μg per ml) or 100 ml of CT (2 μg per ml) were added to wells in ELISA plates. The plates with LPS were placed at 37°C overnight while the plates with CHA or CT were kept in a humid chamber at room temperature overnight. The antigen coated plates were then washed thoroughly with PBS-Tween 20 solution to remove the unbound materials and 150 μl of PBS-Tween 20 containing 0.5% bovine serum albumin were added to

each well to block the sites which were not occupied by the antigens. The plates were kept at 4°C overnight. After another wash as above, 150 µl of serially diluted sera were added to appropriate wells (PBS pH 7.2 was added to the last well of each row in the plates to serve as negative controls). Control positive serum was always included in each experiment. The antibodies in the sera were allowed to react with their appropriate antigens at room temperature for 4 hours then the non-reacted materials washed off by PBS-Tween 20. One hundred and fifty microlitres of 1 : 1,000 peroxidase-labeled antitotal human immunoglobulins or enzyme labeled anti-specific class immunoglobulins (anti-IgG, anti-IgM or anti-IgA; Dako, Denmark) were added to each well. The plates were kept at 4°C overnight. After the final wash, freshly prepared substrate (para-phenylene diamine) in citrate buffer was added. The ELISA end point was determined after 30 minutes by naked eyes then the reaction was stopped by adding 1 N NaOH. The end point was reconfirmed by ELISA reader (Mini Reader II, Dinatech Lab. or Uniskan II, Labsystem).

Statistical analyses: One way analysis of varience (ANOVA) was used to compare means of the ln titres among various age groups. Duncan test was performed to compare between groups of the same study area. Comparisons on the means of ln titres of the same age groups from different study areas were made by student t-test.

RESULTS

Vibriocidal antibody titres in sera of individuals from both study areas against *V. cholerae* 01 reference strain (El Tor,

Table 1

Vibriocidal antibody titres of different age group individuals from H and L areas.

Groups	Vibriocidal titre					Total no. tested
	not detectable at 5^{-1}	5^{-1}	5^{-2}	5^{-3}	5^{-4}	
H area						
1 (newborn - 6 mo)	48*	4**	1	2	0	55
2 (> 6 mo - 1 yr)	28	0	0	0	0	28
3 (> 1 yr - 5 yr)	33	4	4	2	1	44
4 (> 5 - 15 yr)	25	2	12	7	3	49
5 (> 15 yr)	16	12	34	35	6	103
L area						
1	26	12	9	4	0	51
2	12	2	1	0	0	15
3	22	7	4	2	0	35
4	14	6	6	4	0	30
5	9	41	41	15	0	81

mo = month (s)
yr = year (s)
*number of individuals who gave undetectable titre
**number of individuals who gave titre at 1 : 5

Table 2

Means and standard errors of reciprocal ln ELISA titres of anti-LPS.

Ig Class	ln ELISA titres mean ± SE				
"H" area	1 (55)*	2 (28)	3 (45)	4 (49)	5 (104)
T**	5.7 ± 0.1	7.2 ± 0.1	7.5 ± 0.1	7.8 ± 0.1	7.5 ± 0.1
Ig M	4.5 ± 0.2	6.9 ± 0.1	7.2 ± 0.1	7.5 ± 0.1	6.2 ± 0.1
Ig G	5.0 ± 0.1	5.1 ± 0.2	5.8 ± 0.1	6.2 ± 0.1	6.5 ± 0.1
Ig A	1.1 ± 0.2	3.5 ± 0.2	3.6 ± 0.1	4.2 ± 0.2	3.7 ± 0.2
"L" area	1 (62)	2 (18)	3 (39)	4 (37)	5 (85)
T**	6.8 ± 0.1	7.2 ± 0.2	6.9 ± 0.1	7.2 ± 0.1	7.7 ± 0.1
Ig M	5.3 ± 0.2	6.8 ± 0.2	6.3 ± 0.2	6.7 ± 0.1	6.9 ± 0.1
Ig G	5.5 ± 0.2	5.8 ± 0.2	5.5 ± 0.1	5.6 ± 0.1	6.2 ± 0.1
Ig A	1.9 ± 0.2	3.1 ± 0.4	3.3 ± 0.2	3.7 ± 0.2	4.0 ± 0.1

*indicates total subjects tested.
**T = total specific immunoglobulin.

Ogawa, O_{17}SR) are shown in Table 1. Only 13% (7/55) of H and 49% (25/51) of L had detectable maternal transferred vibriocidal antibody (groups 1). However, the passively transferred antibody became undetectable in 100% (28/28) and 80% (12/15) of H and L, respectively, at the age between 6 months to 1 year old (groups 2). Thereafter, the actively acquired antibody by natural means seemed to increase with age i.e. 25% (11/44), 49% (24/49) and 84% (87/103) of individuals from H at the age of groups 3 (> 1 yr - 5 yrs), 4 (> 5 yrs - 15 yrs) and 5 (> 15 yrs), respectively, had vibriocidal titres at 1 : 5 or greater. At the age older than 15 years 40% (41/103) of the people who lived in H had the titre at or more than 1 : 125. Actively acquired vibriocidal antibody of individuals from L also started after 1 year of life, thus, 37% (13/35), 53% (16/30) and 89% (72/81) of individuals of groups 3, 4 and 5, respectively had the titre at 1 : 5 or higher. However, only 19% (15/81) of individuals older than 15 years of age had the titre as high as 1 : 125.

The results on means and standard errors of reciprocal ln ELISA titres of anti-LPS in sera of individuals in H and L are shown in Table 2. The antibody levels in the forms of either total immunoglobulins (T), IgM (M), IgA (A) or IgG (G) in sera of the subjects from L were higher than those from H during the first six months of life (group 1). The means of IgM-anti-LPS detectable at this age group (newborn to 6 months old) were contributed mainly by the antibodies in sera of children aged between 3 to 6 months (Table 3) as it was noted that 15 from 17 of L area, and 10 from 10 of H area gave titres that ranged from 1 : 320 to 1 : 2,560 while younger children (newborn - 1 month old) gave much lower titres. Rapid rises of T, M and A anti-LPS from group 1 to group 2 were noted ($p < 0.01$) from both study areas (table 2). The T anti-LPS of subjects from H reached maximum level at the age of group 3 (1 to 5 years old) and remained at this level until adult life (group 5) while those of subjects from L did not reach the highest

level until adult (group 5). The adult levels of T anti-LPS of H and L were not different ($p>0.01$). The pattern of IgA anti-LPS of L was similar to that of the total immunoglobulins. In contrast, the IgA of H increased rapidly and maximized at the age of group 2 (>6 months to 1 year old). Thereafter, the level was statistically unchanged. The adult level of IgA anti-LPS of H and L were also similar ($p>0.01$). There was no change in the IgM anti-LPS in the sera of subjects from L after the initial rise from the level of group 1 to that of group 2. It was also noticed that the levels of this specific antibody in groups 2, 3 and 4 of subjects from H were significantly higher than those found in the corresponding age groups from L ($p < 0.01$). However, while the adult level of L (group 5) remained as high as in groups 2, 3 and 4, the level in group 5 of H was significantly reduced. The level of IgM anti-LPS in group 5 of H was lower than that of L ($p < 0.01$).

Patterns of specific IgG anti-LPS levels of both H and L were different from the patterns of the antibodies of other classes in that there were no significant difference in the mean levels of group 1 and group 2. Detail analyses of the titres within group 1 from both study areas revealed that during the first month after birth, the IgG levels were high (Table 3). However, the levels gradually decreased and were lowest at the age of 3-6 months old. The overall pattern of IgG levels during the first six months of life, thus, was opposite to the patterns of IgM at the corresponding age group (Table 3) i.e. the IgM levels increased while the IgG titres decreased from those of newborns to 6 months old. The levels of IgG of people from L remained unchanged until the age of group 4, but became highest at the age of group 5. There was a continuous rise in the means of IgG antibody titres of individuals who lived in H from group 2 to group 5. The levels of this class of antibody in group 5 of both areas were not different ($p > 0.01$).

Table 3

Means and standard errors of reciprocal ln IgM and IgG antibody titres against *V. cholerae* LPS of newborn to 6 months old individuals from H and L areas.

Ig class and Age	Titres Mean ± S.E.	
	area H	area L
Ig M		
Newborn - 1 mo	3.4 ± 0.2	4.5 ± 0.5
1 - 3 mo	4.6 ± 0.2	5.8 ± 0.2
3 - 6 mo	6.6 ± 0.2	6.3 ± 0.3
Ig G		
Newborn - 1 mo	5.2 ± 0.2	6.0 ± 0.3
1 - 3 mo	4.8 ± 0.2	5.1 ± 0.3
3 - 6 mo	4.5 ± 0.2	5.0 ± 0.3

The overall patterns of changes in anti-CHA immunoglobulins were similar to those of anti-LPS except that the levels of anti-CHA in group 1 from both study areas were not different (Table 4). It was also observed that while the adult levels of anti-LPS (except IgM) from H and were relatively equal, the levels of anti-CHA from L were higher than from H. The IgA anti-CHA of H rose markedly from group 1 to group 2 while those of L increased rather slowly with age until maximum at group 5.

Results of means and standard errors of serum anti-CT of individuals from H and L are shown in Table 5. Although the levels of all classes of anti-CT of H seemed to increase with age from group 1 to group 3, there was a marked drop in the antibody levels of all classes at the age of group 4 before another rise in group 5. Such a drop was not found in sera from L. The levels of IgA anti-CT were negligible in group 1 of both areas. Significant increase was detected in group 2. Thereafter, the IgA-anti-CT of L remained unchanged while those of H

Table 4

Mean and standard error of ln ELISA titres of anti-CHA in H and L areas.

Ig class "H" area	1 (55)*	2 (28)	3 (45)	4 (49)	5 (104)
Total	6.1 ± 0.1	6.6 ± 0.2	6.8 ± 0.1	6.8 ± 0.1	7.4 ± 0.1
M	5.2 ± 0.1	6.2 ± 0.2	6.3 ± 0.1	6.2 ± 0.1	5.5 ± 0.1
G	5.9 ± 0.1	6.3 ± 0.2	6.4 ± 0.1	6.5 ± 0.1	7.4 ± 0.1
A	1.5 ± 0.2	3.4 ± 0.3	3.3 ± 0.2	3.3 ± 0.2	3.9 ± 0.2
"L" area	1 (62)	2 (18)	3 (39)	4 (37)	5 (85)
Total	6.4 ± 0.1	6.6 ± 0.3	6.6 ± 0.2	6.6 ± 0.3	8.2 ± 0.1
M	4.7 ± 0.2	5.6 ± 0.5	5.6 ± 0.2	5.7 ± 0.2	6.2 ± 0.1
G	6.3 ± 0.1	6.4 ± 0.3	6.3 ± 0.2	6.4 ± 0.3	8.2 ± 0.1
A	1.4 ± 0.2	1.7 ± 0.3	2.4 ± 0.3	3.4 ± 0.3	4.7 ± 0.1

*No. tested.

Table 5

Mean and standard error of ln ELISA titres of anti-CT in study areas H and L.

H area	In ELISA titres				
	1 (55)*	2 (28)	3 (45)	4 (49)	5 (104)
Total Ig	4.7 ± 0.1	5.8 ± 0.2	6.1 ± 0.1	5.7 ± 0.1	6.5 ± 0.1
Ig M	1.0 ± 0.2	4.3 ± 0.2	5.2 ± 0.6	4.5 ± 0.1	4.8 ± 0.2
Ig G	4.3 ± 0.2	4.9 ± 0.2	5.4 ± 0.1	4.9 ± 0.1	5.5 ± 0.1
Ig A	0.1 ± 0.1	1.5 ± 0.3	2.3 ± 0.2	1.4 ± 0.2	2.8 ± 0.1
L area	1 (62)	2 (18)	3 (39)	4 (37)	5 (85)
Total Ig	4.6 ± 0.3	5.2 ± 0.4	5.9 ± 0.2	6.1 ± 0.2	6.8 ± 0.1
Ig M	1.9 ± 0.3	4.0 ± 0.4	5.4 ± 0.2	5.7 ± 0.2	4.9 ± 0.1
Ig G	4.1 ± 0.3	4.4 ± 0.4	4.7 ± 0.2	5.1 ± 0.3	6.5 ± 0.1
Ig A	0.3 ± 0.1	1.5 ± 0.4	1.9 ± 0.3	1.4 ± 0.3	1.3 ± 0.2

*No. tested.

further rose in group 3, dropped in group 4 and reincreased in group 5. A decline of IgM-anti-CT of L in group 5 (similar pattern to IgM-anti-LPS and IgM-anti-CHA of H) was noticeable. Adult levels of anti-CT in the forms of T and G from L were higher than those of H, while IgM levels were similar, and IgA anti-CT of H was higher than that of L (Table 5).

DISCUSSION

Vibriocidal antibody titres were detected in 13% and 49% of infants (age less than 6

months) who lived in areas with high (H) and low (L) cholera morbidity of Thailand, respectively. These maternal transferred antibodies in most infants disappeared at the age between 6 months to 1 year old. Thereafter, the levels of actively acquired antibody through natural antigen stimulation increased with age. Fifty percent of the Thai individuals had detectable vibriocidal antibodies when they are 5 to 15 years old and more than 80% of adults had titres ranging from 1 : 5 to 1:125 or higher. Our results are similar to the studies conducted in Bangladesh, which demonstrated that Bangladeshi individuals acquired vibriocidal antibody early in life and 80% of them were seropositive by 15 years of age (Mosley, 1969). The Thai adults (aged > 15 years) who lived in area of high cholera morbidity had significantly higher vibriocidal antibody titre than their counterparts in the area of low morbidity. This reflects a continuous anamnestic response to natural exposure to vibrio somatic antigens of the people from H. The high vibriocidal titres were found to be associated with protection against cholera (Mosley, 1969; Glass *et al.*, 1985). However, the higher incidence of the disease among the Thai individuals with relatively high vibriocidal antibody titres would be explanable only on the basis that the existing antibody was overcome by the large inoculum of pathogenic vibrios which these people experienced.

Adult levels of anti-LPS of both study areas were not different although the mean vibriocidal titre of individuals from H was higher than that of L. The finding implies that the antigens responsible for the vibriocidal antibody production are not solely LPS.

Levels of IgG anti-LPS in infants (newborns to 6 months) of H were lower than those of their counterparts in L in spite of the fact that the levels in sera of group 5 (adults) were not different. It is unlikely that the difference observed was due to unequal degrees of maternal transferration. It seems possible that the catabolic rate of the passively acquired IgG anti-LPS of individuals from H and L during the first six months of life were different as the influence of frequency and quantity of LPS exposure. The catabolic rate of the transplacentally acquired IgG anti-LPS of individuals from H might be enhanced by the more LPS exposure hence mopping up of the existing antibody.

Active synthesis of IgM anti-LPS of individuals from both areas seemed to start early since the first month of life (Table 3). The lower level of IgM anti-LPS of H than that of L in group 1 may reflect also the mop up of the actively synthesized antibody by the continuously incoming antigen which the individuals of H experienced. The same explanation may also be applied to the IgA anti-LPS.

The levels of IgM and IgG anti-LPS of individuals from H increased steadily from group 1 to group 4. It is reasonable to assume that by 15 years of age (group 5) all of the individuals in H might have had experienced the primary immune response to LPS. Thus, upon re-exposure to the antigen they responded with the IgG (secondary) type of immune response rather than the IgM type. This results in a marked drop of the IgM anti-LPS levels in group 5 of H. This situation might be different for individuals from L in that the people of this study area might have lesser chance of the LPS exposure. The levels of both IgM and IgG anti-LPS, therefore, were not changed so much from group 1 to group 5. Some individuals of L still respond to the antigen with IgM antibody synthesis after 15 years old. As a result, no drop of IgM antibody to the LPS was seen in group 5 of L.

It is difficult to explain why levels of all classes of anti-CHA in group 5 from L were significantly higher than those of their counterparts from H ($p < 0.01$). The levels of T, M

and A of group 1 of both areas were not different ($p > 0.01$) while IgG of L was slightly higher than those of H ($0.01 < p < 0.05$) indicating the more maternal transferred anti-CHA to newborns of L and perhaps, also, the increased removal of the maternal transferred IgG by antigen mopping up in H. A marked decrease of IgM anti-CHA level similar to the drop of anti-LPS in group 5 of H was also seen. It is not known whether the same explanation as has been given for the IgM anti-LPS of H would be applicable for the anti-CHA.

Patterns of anti-CT from individuals of both study areas were quite different from the patterns of the antibodies against LPS and CHA. No explanation could be given on a marked decrease of all classes of anti-CT in H during school age (group 4). Also it is not known why there was a drop of IgM level in group 5 of L. The IgG anti-CT in group 5 of L was significantly higher than that of H. However, the levels of this class of anti-CT in group 1 of both areas were not different. The reason for this finding is also unknown. The higher level of IgA anti-CT in adults of H than that of L may reflect the higher degree of the antigen exposure in the former.

SUMMARY

Vibriocidal antibody and antibodies to *Vibrio cholerae* lipopolysaccharide (anti-LPS), cell-bound haemagglutinin (anti-CHA) and toxin (anti-CT) were determined in Thai individuals of various age groups who lived in areas with high (H) and low (L) cholera endemicity. The enzyme-linked immunosorbent assay (ELISA) was performed to detect levels of class specific anti-LPS, anti-CHA and anti-CT. It was found that Thai individuals acquired the vibriocidal antibody early in life. Fifty percent of individuals aged 5 to 15 years old had detectable titre while more than 80% of adults had titres ranged from 1 : 5 to 1 : 125 or higher. Thai adults who lived in area with high cholera endemicity had significantly higher vibriocidal antibody levels than their counterparts who lived in area with low cholera endemicity. Lipopolysaccharide was not the only antigen responsible for stimulating the vibriocidal antibody production. Adult levels of all classes of anti-CHA from L were higher than those of H while the anti-LPS in the forms of total immunoglobulins, IgG and IgA were similar but IgM of L was higher than that of H. The levels of all classes of anti-CT from H seemed to increase with age except at the school age (5 years to 15 years old) when there were marked decreases of all antibody classes. Total immunoglobulin and IgM anti-CT at adult age of H and L were not different, although IgG anti-CT of L was higher than that of H and IgA anti-CT of H was higher than that of L.

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