SEROLOGICAL ASPECTS OF A CHOLERA TOXOID FIELD TRIAL IN BANGLADESH

George T. Curlin, Richard L. Levine, K.M.A. Aziz, A.S.M. Mizanur Rahman, and Willard F. Verwey

Cholera Research Laboratory, GPO Box 128, Dacca 2, Bangladesh

At the eleventh US-Japan Cholera Panel Meetings, we presented the results of a field trial of a glutaraldehyde cholera toxoid (1). For this symposium we will review briefly the efficacy data of the field trial and present an analysis of the serological data from which we sought evidence of synergism between antibacterial and antitoxin antibodies.

SUMMARY OF THE 1974 FIELD TRIAL

From among the residents of the Cholera Research Laboraotry (CRL) field study area in rural Bangladesh, 92,838 volunteers participated in the field trial. Volunteers were drawn form children between 1 and 14 years of age and adult women. The toxoid, lot number 21201 prepared by Wyeth Laboratories, was a prototype formed by glutaraldehyde treatment of purified cholera enterotoxin obtained from broth cultures of Inaba 569B (2). Volunteers received by jet injector on a double blind basis either 0.5 ml of toxoid (100 mcg) with protamine and aluminium phosphate adjuvants or 0.5 ml of adult-dose tetanus-diphtheria toxoids. Seventy-four percent of the volunteers received a second injection six weeks later. A serological survey was conducted among all volunteers of every thirty-third family immediately prior to the first injection, and six weeks after the first and second injections. Cases were defined as patients who were seen at the CRL hospital located in the field study area with a complaint of diarrhea and from whose stool or rectal swab *V. cholerae* was isolated.

In the following analyses time is expressed as the number of days from the first injection as cholera cases were seen during the vaccination campaign because of the early start of the epidemic and also, as noted previously, one injection and two injections were equally protective.

Table 1 shows the efficacy of the toxoid in protecting against Inaba cholera. The overall protection of the entire year for all ages was 26%, but virtually all of the protection was seen among the 5–14 year-olds during the first 90 days. Although during the first 90 days the rate was lower among toxoid recipients than controls in the other age groups as well, statistically significant protection was seen only in the 5–14 year-olds. Table 2 shows the toxoid efficacy in protecting against Ogawa cholera. No significant protection against Ogawa infection was seen in any age group at any time, but, unlike Inaba infection 65% of which occurred in the first 97 days, 60% of Ogawa infections occurred later in the epidemic. Thus, modest levels of protection of brief duration would not have been detected.

Table 1

Age-Specific Rate/1000 of Inaba Cholera
by Vaccine Group and Interval from First Injection

Interval (days)		Age 1-4	Age 5-14	Age 15+	All Ages
14-97	Toxoid	1.86	0.81	1.12	1.14
	Placebo	2.73	1.99	1.31	1.90
			$p < 0.002$		$p < 0.01$
98-181	Toxoid	0.62	0.20	0.06	0.24
	Placebo	0.53	0.20	0.12	0.24
182-365	Toxoid	0.93	0.66	0.35	0.60
	Placebo	0.84	0.65	0.24	0.54
14-365	Toxoid	3.41	1.67	1.53	1.98
	Placebo	4.10	2.83	1.67	2.67
			$p < 0.02$		$p < 0.05$

Table 2

Age-Specific Rate/1000 of Inaba Cholera
by Vaccine Group and Interval from First Injection

Interval (days)		Age 1–4	Age 5-14	Age 15+	All Ages
14-97	Toxoid	1.86	0.71	0.65	0.93
	Placebo	1.68	1.04	0.54	0.99
98-181	Toxoid	1.24	0.91	0.47	0.82
	Placebo	1.16	0.60	0.30	0.60
182-365	Toxoid	1.55	0.71	0.41	0.78
	Placebo	1.26	0.74	0.42	0.73
14-365	Toxoid	4.65	2.33	1.53	2.52
	Placebo	4.10	2.38	1.25	2.33

SEROLOGICAL ASPECTS OF THE 1974 FIELD TRIAL

Previously we have not commented extensively on the serological aspects of the field trial. However, we feel the serological data deserve a closer look since other investigators have demonstrated synergism between antitoxin and antibacterial antibodies in animal models of experimental cholera (3, 4), and since we observed an unexplained concentration of protection in the field trial among older children, who are characterized as a group by intermediate levels of both vibriocidal and antitoxin antibodies (5).

Serological survey specimens were obtained by finger-stick in the field. The 50 lambda sample was diluted immediately with 450 lambda of sterile saline, and the supernatent was assayed for vibriocidal Inaba antibody by the microtechnique (6) and antitoxin antibody by a hemagglutination assay using purified Finklestein's choleragen to coat the red cells (7). The antitoxin titer was also determined on every fifth sample by the more sensitive rabbit skin assay (8) to confirm the results and extend the lower limits of detectable antitoxin tiers.

As noted in Table 3, the toxoid did not stimulate a rise in vibriocidal titer which we could detect with our assay. As expected from previous reports from Bangladesh (5), higher vibriocidal titers were noted with increasing age.

The percent frequency distribution of antitoxin titers as determined by the rabbit skin assay are given in Table 4. Because of the 1:10 dilution of the specimens, the hemagglutination assay could not detect titers less than approximately 16 antitoxin units per ml (AU/ml). The more sensitive rabbit skin assay routinely detected titers of 8 AU/ml with acceptable correlation between the two assays at higher titers. A higher proportion of individuals with 10 AU/ml or more was noted in the younger ages. This confirms earlier observations of an inverse relationship between age and serum antitoxin among residents of the cholera endemic area of Bangladesh (5). The modest rises in vibriocidal Inaba titer (Table 3) and antitoxin titer (Table 4) which were detected in placebo recipients during the field trial are consistent with our estimate of the overall clinical and inapparent infection rate in the population during the epidemic of 1974.

Table 3

1974 Toxoid Field Trial Serology Survey
Age-Specific Geometric Mean Vibriocidal Inaba Titer

Units/Ml. $\pm$ 1.96 $\sigma/\sqrt{n}$

Age Group	Vaccine Group	Baseline	Day 42	Day 84
1-4 years	Toxoid	2.96 ± 1.10	3.09 ± 1.08	3.62 ± 1.30
	Placebo	3.09 ± 1.08	3.37 ± 1.10	3.78 ± 1.13
5-14 years	Toxoid	6.25 ± 1.10	6.28 ± 1.11	6.84 ± 1.13
	Placebo	6.86 ± 1.10	7.40 ± 1.12	7.02 ± 1.14
15+ years	Toxoid	16.04 ± 1.12	14.49 ± 1.12	14.98 ± 1.18
	Placebo	18.08 ± 1.12	17.03 ± 1.13	15.45 ± 1.19

Table 4

Toxoid Field Trial Serological Survey
Percent Frequency Distribution of Serum Antitoxin* by Vaccine Group and Age

Age Group	Vaccine Group**	Sample Day	<10	10-31	31-99	100-131	>131
1-4	Toxoid	0	73	26	2		
		42	9	16	49	23	2
		84	3	20	66	11	
	Placebo	0	78	18	5		
		42	76	16	5	3	
		84	59	34	3	3	
5-14	Toxoid	0	99	1			
		42	13	30	32	19	6
		84	26	44	26	3	
	Placebo	0	98	2			
		42	91	5	4		
		84	84	14		2	
15+	Toxoid	0	96	4	1		
		42	11	21	25	25	15
		84		20	39	39	2
	Placebo	0	100				
		42	95	3	1	1	
		84	90	2	2	5	

* Rabbit skin assay
** Recipients of two injections

From October 1 through December 31 venepuncture blood for antitoxin and vibriocidal Inaba titers was obtained from all field tiral volunteers admitted with cholera at the CRL hospital. All patients were seen within the first 48 hours of diarrhea and most were seen within the first 12 hours. In the following analyses we assumed the titer on admission accurately reflected the serological status of the patient when the disease was contracted. Data from the third serological survey, which was conducted between October 1 and November 3, was used to calculate the numbers of individuals at risk in each vibriocidal/antitoxin category during the time we sought serological and clinical attack rate correlates.

Table 5 gives the Inaba attack rates for the 1 – 4 year age group as a function of the vibriocidal and antitoxin titers. We have combined data from recipients of two injections of toxoid and placebo. No distinct pattern is seen in this table in which 16 cases are represented. Corresponding rates generated by 21 cases are given for the 5 – 14 year-olds in Table 6. Although the cross tabulation fails to demonstrate any striking pattern the marginals are interesting. Regardless of the vibriocidal titer, the rates for each antitoxin class are very similar. However, while dis-

regarding the antitoxin titer, the rates decrease with elevation in vibriocidal titer. Because only 12 cases of Inaba cholera were seen during this period in adults and none fell into the middle vibriocidal calss, Table 7 was collapsed into two columns. The lack of association between antitoxin titer and attack rate is apparent.

Table 5

Cholera Inaba Rates/1000 by Antibody Titer

Toxoid and Placebo Groups Combined

1 – 4 years

Antitoxin Titer AU/ml	Vibriocidal Inaba units/ml			
	< 6.3	6.3-15.8	> 15.8	Total
< 15.8	1.364	0.000	0.000	1.034
15.8-50.0	0.734	2.857	0.000	0.796
> 50.0	2.006	0.000	2.236	1.792
Total	1.275	0.547	0.734	1.129

Table 6

Cholera Inaba Rates/1000 by Antibody Titer

Toxoid and Placebo Groups Combined

5 – 14 years

Antitoxin Titer Au/ml	Vibriocidal Inaba Units/ml			
	<6.3	6.3-15.8	>15.8	Total
<15.8	1.376	0.282	0.579	0.859
15.8-50.0	0.868	0.947	0.487	0.787
>50.0	0.895	1.956	0.000	0.948
Total	1.109	0.919	0.400	0.755

Table 7

Cholera Inaba Rates/1000 by Antibody Titer
Toxoid and Placebo Groups Combined

15+ years

Antitoxin Titer	Vibriocidal Inaba Units/ml		
AU/ml	< 6.3	> 6.3	Total
< 15.8	1.164	0.460	0.486
15.8-50.0	1.546	0.660	0.653
> 50.0	1.852	0.198	0.454
Total	1.445	0.382	0.496

Table 8

Age-Adjusted Inaba Rates/1000
By Vibriocidal and Antitoxin Titer

Antitoxin Titer	Vibriocidal Titer U/ml			
AU/ml	<6.3	6.3-15.8	>15.8	Total
<15.8	1.281	0.094	0.346	0.793
15.8-50.0	1.049	1.268	0.382	0.745
>50.0	1.584	0.652	0.811	1.065
Total	1.276	0.489	0.505	0.793

As noted earlier, vibriocidal titer, antitoxin titer and attack rate are all strongly age-dependent. Therefore, in an attempt to control for age in combining the data of the previous three tables, Table 8 gives the age-adjusted rates by vibriocidal and antitoxin titer. This does not generate a consistent pattern, although higher rates remain in the lowest vibriocidal classification with the age adjustment.

A statistical analysis of these data can be given in a two-by-two array of vibriocidal and antitoxin titer by comparing the number of cases observed to the number of cases expected if cases were distributed among the population independent of antibody titer.

In Table 9 the comparison between the observed and expected number of cases for the 1 – 4 year age-group is shown. In this youngest age-group few cases were expected in the higher vibriocidal class and no significant association was noted. Among both the 5 – 15 year-olds (Table 10) and adults (Table 11) a statistically significant picture emerged. The pattern is markedly shifted toward higher than expected numbers of cases among those with low vibriocidal titers and a corrsponding lower than expected number of cases observed in the higher vibriocidal classification.

From these analyses we conclude there was no detectable evidence of an additive effect of antitoxin antibodies on the previously documented inverse relationship between vibriocidal titer and attack rate. In fact, there is a conspicuous absence of any consistent pattern of serum antitoxin and attack rate whether one stratifies on the basis of vibriocidal titer or ignores the vibriocidal titer completely. If anything, one observes more cases than expected with high antitoxin titers.

Because we did not alter the vibriocidal titer with the toxoid used in this field trial and, consequently, these data represent naturally occurring vibriocidal antibody, we can not rule out a role of an additive effect of toxoid in a material which contains both toxoid and somatic antigens. Perhaps additive or synergistic effects may occur with simultaneous administration of both types of antigens, and we suggest future field tirals be addressed to answering this theoretically and practically important question.

Table 9

Distribution of Inaba Cases By Vibriocidal and Antitoxin Antibody Titer
Toxoid and Placebo Groups Combined

1 – 4 year age group

Antitoxin Titer AU/ml		Vibriocidal Inaba Titer U/ml <6.3	≧6.3	Total
≦15.8	obs	6	0	6
	exp	4.9675	1.5817	6.5493
>15.8	obs	8	2	10
	exp	7.4299	2.0209	9.4507
Total	obs	14	2	16
	exp	12.3974	3.6026	16.0000

χ^2 = 1.84, N.S.

Table 10

Distribution of Inaba Cases By Vibriocidal and Antitoxin Titer
Toxoid and Placebo Groups Combined

5 — 14 year age group

Antitoxin Tier AU/ml		Vibriocidal Inaba Titer U/ml <6.3	≧6.3	Total
<15.8	obs	8	3	11
	exp	4.5890	5.0811	9.6701
≧15.8	obs	6	4	10
	exp	4.9385	6.3914	11.3299
Total	obs	14	7	21
	exp	9.5275	11.4725	21.0000

$\chi^2 = 4.51$ $p < 0.05$

Table 11

Distribution of Inaba Cases By Vibriocidal and Antitoxin Titer
Toxoid and Placebo Groups Combined

15+ year age group

Antitoxin Titer AU/ml		Vibriocidal Inaba Titer U/ml <6.3	≧6.3	Total
<15.8	obs	3	3	6
	exp	1.2775	4.8385	6.1160
≧15.8	obs	4	2	6
	exp	1.1234	4.7607	5.8840
Total	obs	7	5	12
	exp	2.4009	9.5991	12.0000

$\chi^2 = 11.99$ $p < 0.005$

REFERENCES

1. Curlin, G. T., R. Levine, K. M. A. Aziz, A. S. M. M. Rahman, and W.F. Verwey: Field trial of cholera toxoid, "Proceedings of the Eleventh Joint Conference on Cholera", pp. 314-329, 1975, the U.S.-Japan Cooperative Medical Science Program.
2. Rappaport, R. S., B. A. Rubin, and H. Tint: Development of a purified cholera toxoid. 1. Purification of toxin. *Infect. Immun.* 9: 294-303, 1974.
3. Peterson, J. W.: Personal communication.
4. Svennerholm, A. and J. Holmgren: Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. *Infect. Immun.* 13: 735-740, 1976.
5. Woodward, W. E., W. H. Mosley, and W. M. McCormack: The spectrum of cholera in rural East Pakistan. 1. Correlation of bacteriologic and serologic results. *J. Infect. Dis. 121* (supplement): S10-S14, 1970.
6. Benenson, A. S., A. Saad, and W. H. Mosley: Serological studies in cholera. 2. The vibriocidal antibody response of cholera patients determined by microtechnique. *Bull. W.H.O. 38:* 277-285, 1968.
7. Ahmed, A., A. Mahmood, P. K. B. Neogi and G. T. Curlin: Serological aspects of a field trial of cholera toxoid. 1. Description of a passive hemagglutination technique for estimation of cholera antitoxin. (munuscript in preparation).
8. Craig, J.P.: A permeability factor (toxin) found in cholera stool and culture filtrates and its neutralization by convalescent cholera sera. *Nature 207:* 614-616, 1965.

DISCUSSION

Dr. Mosley: I think you showed pretty convincingly the antitoxic titers are not related to any protection at least after 60 days or more. Let me make a comment on a simplification in the analysis based on an experience of vibriocidal titers, a graph that Dr. Benenson showed earlier. In fact, in the immunized population with antibacterial vaccine, you raised the titers but you moved them down the same curves, so that age effect of protection apparently is identical to the titer effect of protection in the endemic area. What this means is in the sence that you are combining your data but you do not need to adjust for age because the age effect seems to be the titer effect. I think you could combine your data of all ages simply by distribution of vibriocidal titer and by distribution of antitoxin titer. I am sure you will have the same results.

Dr. Curlin: You are right. I think it is a problem getting enough people with high titers, young kids with high vibriocidal titers, vice versa with antitoxins. I am not sure all the age effects are in fact serological. Most data presented here are stratified by age.

Dr. Benenson: There is another age effect. That is the fact that the young people had relatively high levels of antitoxin but this disappeared with age.

Dr. Mosley: If you graph the age per attack rate by vibriocidal antibody titer, you get very nice straight line, where the titers produced by age or by vaccine are 10^4 very neatly, and correlation was very strong. One thing which does happen is that youngest children have the highest naturally appearing antitoxic antibody but also have the highest case rate in the endemic area without vaccination. Therefore, if you just look at that naturally occurring antitoxin titers and case rate, you can find out that there is inverse correlation, the highest naturally acquired antitoxin titers are related to the highest case rate.

Dr. Curlin: The antitoxin is only thing manupulated in the data presented today, so that antitoxin analogy to age ajustment is more likely than vibriocidal. If I did not age-adjust, there is no way to get children and adult on the same line.

Dr. Sack: If you have a group of the highest antitoxin titer, do you still see any correlation, or you have very high titers at the time they got cholera?

Dr. Curlin: Unfortunately we have too many and very highest titer group have cholera. There is no correlation. We have pateints' sera having the antitoxin titer more than 350 u/ml.

Dr. Finkelstein: My question is about any possible differences between previous Wyeth glutaraldehyde toxoid and present one, which is relatively ineffective in the field. We know about vibrio lipopolysaccharide is large molecular aggregate and offer protection against gut challenge when it is administered parenterally. Dr. Fujita in my laboratory some years ago, indicated his results that procholeragenoid which is a huge molecular aggregate of cholera toxin antigen, when administered parenterally gave better gut immunity than other forms of toxin antigen administered parenterally. Now in the preparation of the Wyeth toxoid, these have gone through variety of evolutionary changes and we found finally in 1975 that they were able to remove the last residual traces of lipopolysaccharide which should have been removed much earlier, by the step of membrane ultrafiltration. Now I saw in R. Rappaport's data that the glutaraldehyde treatment produced a variety of molecular size species of toxin antigen from very large to medium to normally sized toxin antigen. Is it not possible that the last step of Sartorius membrane filtration removed the large molecular aggregates of glutaraldehyde toxoid and left small which may not have a good gut protective effect.

Dr. Gill: I cannot say anything about the upper size but there is nothing in as glutaraldehyde toxoid which si of the same size as formaldehyde toxoid. Formol toxoid is monomeric and everything in glutaraldehyde toxoid is larger than that.

Dr. Pierce: I think the answer is simply by stating that ultrafiltration step is placed for toxoiding. Toxoiding was done after ultrafiltering so ultrafiltrate of toxin does not have any these aggregated materials.

Dr. Finkelstein: Is it then true that new lot is not a derivative of old lot that has been subsequently processed to remove the residual endotoxin after toxoiding?

Dr. Craig: No, these are two separate lots. If I gave the impression that lot 10201 is a precursor of 20101, I am sorry. I have to make one correction that at least two years before the field trial this process was known to remove the last residues of somatic antigen.

Dr. Finkelstein: Staphylococcal β-toxoid, aggregated forms are found to be better protective in recent publication. Additionally, polymeric forms of antigen are known to be T cell-independent and stimulate largely B cell.

Dr. Verwey: In talking about this field trial, I would like to caution about one thing. This field trial was carried on during a period of extreme food abbrevation and so forth. Under those circumstances it is well known that a nutritional state does do any different things or not to show about in the gut. Now you think ordinarily that will be canceled out by control group and so forth. But if there is an interaction between the nutritional state of the person and what he does in regard to the antibody, this should, we say, increase susceptibility conceivably, and must be taken into consideration in this figure, whether one adjust age or not. I don't know if this is true but caution to look for the kind of things.

Proceedings of the 12th Joint Conference
U.S. - Japan Cooperative Medical Science Program
Cholera Panel

SYMPOSIUM ON CHOLERA
SAPPORO, 1976

Edited by

Hideo Fukumi and Yutaka Zinnaka

1977

Japanese Cholera Panel
U.S.-Japan Cooperative Medical Science Program
National Institute of Health
Tokyo 141, Japan