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PREFACE

The Cholera Research Laboratory (CRL) operates under a bilateral project agreement between the government of Bangladesh and the United States of America. Research activities of CRL center on the interrelationships between diarrheal disease, nutrition, fertility and their environmental determinants. CRL issues two types of papers: scientific reports and working papers which demonstrate the type of research activity currently in progress at CRL. The views expressed in these papers are those of authors and do not necessarily represent views of Cholera Research Laboratory. They should not be quoted without the permission of the authors.

ABSTRACT

A gluteraldehyde-treated prototype cholera toxoid lot No.21201 by Wyeth Laboratories was field-tested on 92,838 participants from field study area of the Cholera Research Laboratory in rural Bangladesh. Volunteers received on a double blind basis either 0.5 ml of toxoid (100 mcg) with protamine and aluminum phosphate adjuvants or 0.5 ml of adult dose Diphtheria-tetanus toxoid. Seventy-four percent of them received a second dose, six weeks later. A serological survey was conducted among all volunteers of every thirty third family by taking finger-stick blood in saline (1:10) immediately prior to the first injection, and six weeks after the first and second injections.

The supernate of blood sample was assayed for vibriocidal Inaba antibody by microtechnique and antitoxin antibody by a passive hemagglutination specially developed at CRL. Antitoxin titer was also determined on every fifth sample by rabbit skin assay for coroboration. Cases were defined as patients who were seen at the CRL field hospital with a complaint of diarrhea whose stool or rectal swab yielded V. cholerae on culture.

A brief review of the efficacy data of the field trial, that was presented at the 11th U.S.-Japan Joint Conference on Cholera, is given which shows that in Inaba cholera the overall protection of the entire year for all ages was 26%, but almost all of the protection was restricted among the 5-14 year-olds during the first 90 days, while in case of Ogawa cholera no significant protection was seen in any age group in any time. As 60% of Ogawa infections occurred in the later part of the epidemic, modest levels of protection of brief duration would not have been detected.

The toxoid did not stimulate a rise in vibriocidal titer but, as expected from previous reports from Bangladesh, higher titers were noted with increasing age.

Due to initial one extra two-fold dilution of the plasma the passive hemagglutination assay for antitoxin quantification proved to be less sensitive than the rabbit-skin assay. The data of skin assay shows that a higher proportion of individuals with 10 AU/ml or more was noted in younger ages confirming earlier observations of an inverse relationship between age and serum antitoxin among residents of endemic areas of Bangladesh. A modest rise in vibriocidal Inaba

titer and antitoxin titer that was detected in the placebo participants was accountable for the estimate of overall clinical and inapparent infection rate in epidemic of 1974.

To seek serological and clinical attack rate correlates by calculating the numbers at risk in each vibriocidal/antitoxin categories no distinct pattern was found in 1-4 year age group. In 5-14 year age group attack rate had no correlation with antitoxin titers, however, while disregarding the antitoxin titer, the rates decrease with elevation in vibriocidal titer. To obviate the strong age-dependency of vibriocidal titer, antitoxin titer and attack rate an examination of age - adjusted attack rates as a function of vibriocidal and antitoxin titer did not show any consistent pattern, although higher rates remained in the lowest vibriocidal class.

A statistical analysis of these data when arranged 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, no significant association was noted in 1-4 year age group; but, significant patterns of lower than expected number of cases in higher vibriocidal classes and vice versa were observed in both the 5-14 year and adult groups.

A future field testing of a vaccine containing both toxoid and somatic antigens is suggested in which we might see the additive effect of both the antibodies on attack rates.

At the eleventh U.S.-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 Laboratory (CRL) field study area in rural Bangladesh, 92,838 volunteers participated in the field trial. Volunteers were drawn from 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.

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 OGAWA 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 microliter blood sample was diluted immediately with 450 microliter of sterile saline, and the supernatant was assayed for vibriocidal Inaba antibody by the microtechnique (6) and antitoxin antibody by a passive hemagglutination assay using Dr. Finkelstein's purified choleragen (lot: 0572) to sensitize human erythrocytes with the help of gluteraldehyde as a coupling reagent (7). The antitoxin titer was also determined on every fifth sample by the more sensitive rabbit skin assay (8) to confirm the results of antitoxin assay by hemagglutination and extend the lower limits of detectable antitoxin titers.

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 as determined by the rabbit skin assay are given in Table 4. Because of the 1:10 initial dilution of the specimens in the field and subsequent 1:20 dilution in the first well of the micro-passive hemagglutination assay, the lowest limit of estimation of anti-cholera-toxin by this hemagglutination assay in a couple of titrations (2 runs in 53 titrations) went up to approximately 16 AU/ml; though the mean value was 8.09 AU/ml (SD = $\pm$ 2.6). The rabbit skin assay came out to be more sensitive due to the use of only the 1:10 initially diluted samples and routinely detected titers of 8 AU/ml. The hemagglutination data showing correlations with those of the skin assay will be shown elsewhere (7). Table 4 shows that 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

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 $\pm$ 1.10	3.09 $\pm$ 1.08	3.62 $\pm$ 1.30
	Placebo	3.09 $\pm$ 1.08	3.37 $\pm$ 1.10	3.78 $\pm$ 1.13
5-14 years	Toxoid	6.25 $\pm$ 1.10	6.28 $\pm$ 1.11	6.84 $\pm$ 1.13
	Placebo	6.86 $\pm$ 1.10	7.40 $\pm$ 1.12	7.02 $\pm$ 1.14
15+ years	Toxoid	16.04 $\pm$ 1.12	14.49 $\pm$ 1.12	14.98 $\pm$ 1.18
	Placebo	18.08 $\pm$ 1.12	17.03 $\pm$ 1.13	15.45 $\pm$ 1.19

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.

From October 1 through December 31 venipuncture blood for antitoxin and vibriocidal Inaba titers was obtained from all field trial 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

TABLE 4

TOXOID FIELD TRIAL SEROLOGICAL SURVEY, PERCENT FREQUENCY DISTRIBUTION OF SERUM ANTITOXIN* BY VACCINE GROUP AND AGE

Age Group	Vaccine Group**	Sample Day	Serum Antitoxin AU/ml <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

TABLE 5

CHOLERA INABA CASE RATES/1000 BY ANTIBODY TITER
TOXOID AND PLACEBO GROUPS COMBINED

1 - 4 years

Antitoxin units/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

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 disregarding 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 class, Table 7 was collapsed into two columns. The lack of association between antitoxin titer and attack rate is apparent.

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

TABLE 6

CHOLERA INABA CASE RATES/1000 BY ANTIBODY TITER
TOXOID AND PLACEBO GROUPS COMBINED

5 - 14 years

Antitoxin Units/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 CASE RATES/1000 BY ANTIBODY TITER
TOXOID AND PLACEBO GROUPS COMBINED

15+ years

Antitoxin Units/ml	Vibriocidal Inaba Units/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 CASE 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

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 corresponding lower than expected number of cases observed in the higher vibriocidal classification.

From these analyses we conclude that 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 cannot rule out a

TABLE 9

DISTRIBUTION OF INABA CASES BY VIBRIOCIDAL AND ANTITOXIN ANTIBODY TITER TOXOID AND PLACEBO GROUPS COMBINED

1 - 4 year age group

Antitoxin Titer		Vibriocidal Inaba Titer U/ml		
AU/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

$X^2 = 1.84$, N.S.

TABLE 10

DISTRIBUTION OF INABA CASES BY VIBRIOCIDAL AND ANTITOXIN TITER TOXOID AND PLACEBO GROUPS COMBINED

5 - 14 year age group

Antitoxin Titer		Vibriocidal Inaba Titer U/ml		
AU/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

$X^2 = 4.51 \quad p < 0.05$

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	Vibriocidal Inaba Titer U/ml ≥ 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

$X^2 = 11.99 \quad p<0.005$

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 trials be addressed to answering this theoretically and practically important question.

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