

BODY OF REPORT

SEATO Medic Study No. 70 Pathogenesis and Immunology of Cholera

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Objective: The objective of this study is to further our understanding of the pathogenesis and immunology of cholera.

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Description: This new SEATO study is essentially a continuation of previous work by the Principal Investigator and his colleagues at WRAIR. Previously, it was found that a potent choleraenic product, designated "choleraenic", is elaborated by Vibrio cholerae (strain 569 B, Inaba) during growth in a synthetic (chemically defined) medium supplemented with casamino acids ("Syncase medium"). This product causes an experimental disease resembling human cholera in infant rabbits following its oral, but not parenteral, administration. It has also been demonstrated (Chanyo Benyajati, Brit. Med. J., in press) to produce a cholera-like diarrhea in human volunteers. Choleraenic has been shown to consist of (a minimum of) two components, Procholeraenic A and Procholeraenic B, both of which are required for the production of experimental cholera. Procholeraenic A is a heat labile, non-dialyzable, antigenic component, while Procholeraenic B is heat stable and dialyzable. Up to this time, Procholeraenic A exists in mixture with cholera endotoxin, nucleic acids and other non-dialyzable constituents and products of the cells. It has been established that cholera endotoxin, either by itself or in combination with Procholeraenic B, does not cause experimental cholera. Procholeraenic B, admixed with other small molecular components present initially in the medium and produced by the vibrios during growth. Antisera against choleraenic neutralized its choleraenic activity when mixed with choleraenic prior to administration to infant rabbits and have some protective effect against intra-intestinal infection with living vibrios. However, the same antisera had no protective effect when they were administered parenterally prior to choleraenic (given per os into the stomach). Antisera produced against live vibrios had no neutralizing activity against choleraenic, but were somewhat protective against the infection.

This study was designed to consist essentially of four parts or phases of investigation which are intended to proceed more or less simultaneously, each part supplementing the others:

1. Isolation and purification of the components of choleraenic.
2. Development of an immunologic assay for choleraenic including immunologic studies of experimental cholera induced by choleraenic or by infection.
3. Study of the mode of action of choleraenic and pathogenesis of cholera infection in experimental animals.
4. A collaborative study of the mode of action of choleraenic and pathogenesis of cholera infection in volunteers and in natural cases of cholera.

In addition, bacteriological support is furnished to the Thai Department of Health. Since 25 February, some 337 rectal swabs and 128 water samples have been tested for the presence of cholera vibrios in an effort to promptly recognize carriers, cases and infected water to forestall a serious cholera outbreak.

Progress: Attempts to develop procedures for the isolation and purification of choleraenic have revealed that the choleraenic activity is removed by adsorption

Table I
EFFECT OF KAOLIN, CHARCOAL OR FORMALIN TREATMENT ON
CHOLERAGENICITY AND ENDOTOXIC ACTIVITY OF
SYNCASE CHOLERAGEN

Treatment of Syncase Choleraegen	Endotoxic Activity					Choleraegenicity
	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	
Kaolin (k)	-	-	8/8*	7/8	3/8	0/3**
Charcoal (c)	0/7	1/8	0/8	1/8	1/8	0/3
Mixture of k and c						
Treated Supernates	-	-	-	-	-	0/4
Formalin, 0.5%			8/8	7/8	0/7	0/4
Formalin, 0.25%			8/8	8/8	2/7	0/2***
Formalin, 0.1%			7/8	6/8	0/8	3/3
None (Syncase choleraegen control)			15/16	15/16 m	1/15	21/21

* No. of 11 day check embryos dead/total inoculated intravenously with 0.1 ml of indicated dilution of treated Syncase choleraegen.

** No. of infant rabbits manifesting choleraic diarrhea/total fed 1.0 ml of treated choleraegen per 100 gm body weight following gastric lavage.

*** Fluid, slightly in excess of normal, was noted in the intestines of both animals at time of sacrifice at 18 hours.

with kaolin or activated charcoal. As indicated in Table I, the endotoxic activity also is removed by charcoal, but not by kaolin. In attempts to alter the course of the experimental disease, infant rabbits were fed kaolin or charcoal following the administration of choleraegen. However neither kaolin nor charcoal affected the outcome; the experimental rabbits still succumbed to the choleraic diarrhea. These results are interesting in view of the fact that treatment with kaolin has been reported to be highly beneficial to cholera patients. Perhaps insufficient amounts of the adsorbants were used in these experiments or the choleraegen was already "fixed" to the substrate in the intestinal tract. If choleraegen is actually involved in the natural disease in humans, it might be continually liberated by the vibrios in the intestinal tract. An absorbant might compete for it with the tissues of the host to be benefit of the patient. The subject merits further consideration.

In an attempt to determine its stability under different conditions of pH, crude Procholeraegen A was suspended in tris-hydroxymethyl-methylamine buffers at varying pH levels and incubated at 37 C for 4 hours and, in parallel, overnight under refrigeration. Following this treatment, Procholeraegen B was added and the mixtures fed to infant rabbits. Under these conditions in buffer solutions of pH 4 to pH 10, the choleraegenic activity appeared to be stable. Some inactivation was noted in the pH 2 buffer under both incubation conditions.

The choleraegenicity of choleraegen is inactivated by treatment with 0.5% formalin which leaves the endotoxin activity (also Table I). Some choleraegen remains

active after treatment with lower concentrations of formalin. These results offer some promise for the eventual production of an oral immunogen against the cholera. As our previous immunological observations have suggested, it may be necessary to stimulate local antibody production to attain effective immunity against the cholera.

Further evidence for the local site of cholera action was gained in an experiment in which cholera was administered per os into the stomach of infant rabbits whose intestinal tract had been separated from the stomach by duodenal transection and ligation. None of the animals developed experimental cholera although sibling sham-operated controls manifested the disease in the usual fashion.

The question of whether cholera is primarily a cellular constituent or, rather, a product excreted by the vibrios is of some interest. Certainly, some cholera may be found in the bacterial cells as demonstrated by Dutta and his colleagues and confirmed by us. However, demonstration of intra-cellular cholera activity requires the administration of multiple doses of cell juice representing rather enormous numbers of cholera vibrios. In contrast, filtrates of cholera cultures (such as Syncase cholera or brain heart infusion broth culture filtrate) are cholera in the infant rabbit when administered in a single dose. Further evidence that the cholera is primarily extracellular was obtained by the demonstration (Table II) that neutralizing antiserum against Syncase cholera retained its neutralizing activity following exhaustive adsorption with viable cholera vibrios. Further, antisera against cholera produce several bands of precipitation when

Table II

EFFECT OF ADSORPTION WITH LIVING VIBRIOS ON THE NEUTRALIZING ACTIVITY OF ANTISERUM VS SYNCASE CHOLERA

<u>Treatment of Syncase cholera*</u>	<u>Results</u>
Antiserum vs Syncase cholera	0.6**
Antiserum vs Syncase cholera ads. with living vibrios***	0/12 13/14
None (Syncase cholera control)	13/14

* Serum (or saline, in control) was mixed with Syncase cholera in the proportion of 1:10 one hour prior to feeding infant rabbits.

** No. of animals responding with choleraic diarrhea/total.

*** Growth from 10 Kolle flasks of meat extract agar was used to adsorb 5 ml of antiserum.

tested against crude cholera toxin by the Ouchterlony agar well double diffusion technique. The same antiserum, adsorbed with viable vibrios produces a single band of precipitation with cholera toxin reflecting the reaction between cholera toxin and its specific antibody. Antisera against living vibrios, which did not neutralize cholera toxin, did produce bands of precipitation with antigens contained in crude cholera toxin, but the bands were not identical with the specific cholera toxin-anti-cholera toxin band.

The Ouchterlony test has proven useful as an assay technique in the separation and purification of the Procholera toxin A moiety from the Procholera toxin A fraction which contains a variety of other substances and which includes the cholera endotoxin which can be demonstrated by its toxicity on intravenous inoculation of 11 day chick embryos. Although we have previously shown that cholera endotoxin does not cause experimental cholera, either by itself, or in mixture with Procholera toxin B, we had not eliminated the possibility that endotoxin is required in addition to a heat labile constituent of the Procholera toxin A fraction. In the experiment to be described, concentrated Procholera toxin A was applied to a Sephadex G-200 column and eluted with distilled water. Consecutive fractions were collected and tested for cholera toxinicity (on mixture with Procholera toxin B) in infant rabbits; for endotoxin activity in the chick embryo; for antigen active in the vibriocidal antibody inhibition test (VAIT) (Finkelstein, J. Immunol., 89: 264, 1962); and in the Ouchterlony test using specific anticholera toxin serum and, in parallel, live vibrio antiserum which precipitated cholera endotoxin. The results are summa-

Table III

SEPARATION OF PROCHOLERA TOXIN A AND ENDOTOXIN ON SEPHADEX G-200
SEPHADEX FRACTION

	5	6	7	8	9	10	11	12	13	14	15	16	17
Pptn band with anti-cholera toxin	-	-	-	-	-	-	-	±	++	++	±	-	-
Cholera toxinicity	-	-	-	-	-	-	-	±	++	++	±	-	-
Endotoxin pptn.	-	+	++	+	-	-	-	-	-	-	-	-	-
Endotoxin activity (chick embryo)	-	+	++	+	±	±	tr	tr	n.t.	n.t.	n.t.	n.t.	n.t.
Hapten pptn.	-	-	-	-	-	-	±	±	±	±	±	-	-
VAIT activity	-	+	++	+	+	±	tr	tr	tr	tr	tr	tr/2	tr/2

rized in Table III. The data indicate that the cholera activity was present primarily in fractions 12, 13, 14 and 15 which also have specific precipitation with anti-cholera antibody and which were essentially free of the cholera endotoxin. The latter was primarily concentrated in the earlier fractions as demonstrated by the chick embryo toxicity, by precipitation with live vibrio antiserum and by the VAIT activity. Some hapten apparently was present in the cholera activity fractions as demonstrated by the slight precipitation with the live vibrio antiserum (distinct from both the endotoxin and the cholera bands) and slight activity in the VAIT.

The results indicate that Procholera A is quite an active substance. A minimal dose of Syncase cholera, which would be cholera and lethal for the infant rabbit on oral administration, has been calculated to contain approximately 68 μ g of purified Procholera A. Based upon Dr. Chanyo's experience with human volunteers, a dose which would be cholera in man would contain approximately 54 mg of Procholera A, at the present state of purity.

In the initial stages of an effort to determine the essential components of the casamino acid mixture added to the synthetic medium base for cholera production, amino acid analyses indicated that more than 50% of the following amino acids were utilized during growth of the vibrios in the Syncase medium: aspartic acid, threonine, serine, glutamic acid, tyrosine, arginine and lysine. These amino acids will be incorporated at appropriate levels in the basal medium to determine whether cholera production can be obtained in a completely defined medium.

Assistance has been rendered to the Thai Department of Health in their efforts to avert a severe cholera outbreak. In the period from 25 February to 18 March, 337 rectal swabs and 128 water samples from Bangkok, Thonburi, Paknam, Samutprakan and Pathumthani were tested for the presence of cholera vibrios. Rectal swabs, placed in alkaline peptone water, were streaked as soon as received on meat extract agar (pH 7.6) and alkaline lauryl sulfate tellurite agar. The plates were examined by the oblique light technique at 8-12 hours and suspicious colonies tested by slide agglutination in cholera grouping serum. If positive, they were typed by slide agglutination in type specific sera, Inaba and Ogawa, and tested for hemagglutination by the procedure of Finkelstein and Mukerjee (Proc. Soc. Exp. Biol. Med. 112:355, 1963). If negative, the peptone water enrichment, which had been maintained at 37 C, was re-streaked and the procedure repeated. Water samples were processed by passing approximately 100 ml through Millipore filters. The pad was then incubated in alkaline peptone water for 6-8 hours; this enrichment culture then being treated in the same manner as the rectal swabs, above. Of the rectal swab cultures, 9 yielded cholera vibrios of the El Tor Inaba type. Five of these strains were from healthy contact carriers, while 4 were from cases. Their identity as El Tor vibrios was confirmed by tests which revealed their exalted virulence for chick embryos (Finkelstein, Nature, 202:609, 1964). Six of the strains, from both contacts and cases, were tested for cholera activity in infant rabbits by intra-intestinal inoculation with inocula of the order of 10^4

vibrios. All were found to be choleraogenic in this test regardless of whether they were isolated from patients or carriers. No cholera vibrios were isolated from any of the water samples received for testing. However, two non-agglutinable (in cholera sera) vibrio strains, of Heiberg I fermentative characteristics, which resembled cholera vibrios in their colonial appearance, were isolated. These two strains were hemagglutinative and exhibited exalted virulence for chick embryos, but were not choleraogenic in infant rabbits and were found to be unsusceptible to all of Mukerjee's cholera and El Tor phages in our hands. Attempts to differentiate the 1965 El Tor vibrio isolates from 1964 strains by phage typing using Mukerjee's El Tor phages yielded equivocal results because of the low titers of our El Tor phage stocks.

Summary: It has previously been demonstrated by the Principal Investigator and his colleagues that V. cholerae (strain 569 B, Inaba) elaborates a potent choleraogenic principle, cholera toxin, in a simple defined medium supplemented with cassamino acids (Syncase medium). Cholera toxin causes a fatal experimental disease resembling cholera in infant rabbits following oral administration and has been shown (Benyajati, Brit. Med. J., in press) to cause a cholera-like disease in human volunteers. It can be separated into two components, Procholera toxin A and Procholera toxin B, by dialysis. Both are required for the production of experimental cholera. Procholera toxin A has been obtained in a partially purified form, essentially free of cholera endotoxin, by means of gel filtration with the "molecular sieve", Sephadex G-200. The choleraogenic activity of Syncase cholera toxin is removed by treatment with kaolin or with activated charcoal. It is also inactivated by treatment with formalin which might be a step in the direction of an oral vaccine against the cholera toxin. Procholera toxin A is stable over a fairly broad pH range, but appears to be partially inactivated at pH values below 4. The accumulative evidence suggests that cholera toxin acts locally and superficially at the surface of the intestinal mucosa to produce the diarrhea of experimental cholera. Antisera against live vibrios did not neutralize the choleraogenic activity of Syncase cholera toxin which is neutralized by anti-cholera toxin antisera even following their adsorption with viable vibrios. The adsorbed sera specifically precipitate purified Procholera toxin A in a single band in the Ouchterlony test. In the early stages of an effort to produce cholera toxin in completely chemically defined medium, the amino acids which are utilized by the cholera vibrio during growth in the Syncase medium have been identified. Strains of El Tor cholera vibrios isolated from both patients and carriers in the Bangkok area in 1965 were found to be choleraogenic in infant rabbits.

Conclusions: An immunologic system for recognition of Procholera toxin A has been developed. Procholera toxin A has been obtained in a purified form essentially free of cholera endotoxin. The amino acids which are utilized by V. cholerae during growth and cholera toxin production in Syncase medium have been identified. The choleraogenic activity of Syncase cholera toxin is removed by treatment with kaolin or charcoal. The latter, but not the former, removes the endotoxin activity as well. Cholera toxin is also inactivated by formalin. It is fairly stable over a wide pH range but appears to be inactivated in buffer of pH less than 4.0. Strains of

El Tor Inaba Vibrios recently isolated from both patients and healthy contact carriers in Thailand were found to be choleraogenic in the infant rabbit.

Publications: Finkelstein, R.A., Powell, C.J. Jr., Woodrow, J.C. and Krevans, J.R. Serological responses in man to a single small dose of cholera vaccine with special reference to the lack of influence of ABO blood group on natural antibody or immunological responsiveness. Bull. Johns Hopkins Hosp., 116:152, 1965.

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