

SEATO MEDICAL RESEARCH STUDY ON CHOLERA

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

Description—Efforts during the year have been directed primarily to an expansion of our knowledge concerning the production, mode of action, and immunology of "cholera toxin," an antigenic, cholera toxin, protein moiety elaborated in vitro by certain strains of cholera vibrios. In addition, an effort was made to develop a more effective conventional cholera vaccine based upon a new method of examining the antigenicity of candidate strains.

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Progress:

1. Production of cholera toxin in a simple, completely defined medium.

The present method for the production of cholera toxin involves the growth of *V. cholerae* 569 B in a synthetic medium supplemented with casamino acids, an un-defined mixture of amino acids derived from the hydrolysis of casein. This apparent requirement of casamino acids has been confirmed in several laboratories. If cholera toxin could be produced in a completely defined medium, it might facilitate the ultimate purification of this material and it might, at the same time, enable the production of cholera toxin in greater yield. We had previously attempted to substitute a mixture of amino acids, based on an analysis of the casamino acid medium, but failed to obtain detectable cholera toxin production. In view of the present importance of this problem, it was decided to attempt again the production of cholera toxin in a completely defined medium. Accordingly, a battery of media was prepared consisting of the simple basal medium with various supplements; a mixture of the L-amino acids found in casamino acids (we had previously used DL-amino acids), ash from casein, a vitamin mixture, a complete tissue culture medium, and various combinations of the supplements. *V. cholerae* 569 B was cultivated in the various media in the usual manner (with an inoculum of 10^3 – 10^4 cells per ml at 37 C in fairly shallow layers with shaking) and filtrates were prepared and tested for cholera toxinogenicity in infant rabbits. It was found that substitution of a mixture of 16 L-amino acids for casamino acids allowed production of cholera toxin in the basal medium. The following amino acids and amounts were used:

<u>Amino acid</u>	<u>mgm%</u>
Aspartic acid	19
Threonine	12.5
Serine	16
Glutamic acid	59
Proline	20
Glycine	7
Alanine	10
Valine	17.5
Methionine	8
Isoleucine	12
Leucine	30
Tyrosine	5
Phenylalanine	11.5
Lysine	30
Histidine	10
Arginine	12

Subsequent tests, employing pools of individual amino acids, have yielded variable and inconsistent results. Additional study to determine the optimal conditions for satisfactory yields of cholera toxin is in progress.

The Department of Biologics Research, WRAIR, has undertaken the production of larger batches of crude cholera toxin which have been forwarded to this laboratory for further purification. Thus far, four lots have been received and three of these have been processed. The first was relatively inactive, but purified cholera toxin has been obtained from the other two processed. The yields have been lower than might be expected from previous experience with pools of small batches of crude filtrate.

The majority of cholera strains which have been tested failed to elaborate cholera toxin in vitro (under the conditions we employ for cholera toxin production from strain 569 B) although most of the same strains are of proven cholera toxinogenicity in the intra-intestinally infected infant rabbit. To test the possibility

that failure to elaborate detectable cholera toxin in vitro may be due to its inactivation or breakdown by non-elaborating strains, a known amount of cholera toxin (20 ug/ml) was added to a Syncase broth culture of a non-elaborating strain (NIH 35, Inaba) and, in parallel, to sterile medium and to a culture of 569 B. After 18 hours at 37°C, filtrates were prepared and tested in infant rabbits. The culture filtrate from NIH 35 without added cholera toxin was, as expected, not cholera toxinogenic; the filtrate with added cholera toxin was cholera toxinogenic as were the filtrates of sterile medium with added cholera toxin and the filtrate of 569 B with cholera toxin. Thus, tests with this single strain suggest that in vitro inactivation of cholera toxin is not the reason for the failure of "non-elaborating" strains to produce cholera toxin in vitro.

2. Further purification of cholera toxin on ion-exchange resins.

Previous study of "purified cholera toxin" (cholera toxin which was passed through Sephadex) by disc electrophoresis indicated that multiple protein components were present: as many as 11 protein-staining bands could be detected by this sensitive technique, with only one area associated with cholera toxin antigen as demonstrated by immune precipitation. Accordingly, an attempt was made to further purify cholera toxin by means of ion exchange. A 5 mg sample of cholera toxin was applied to a DEAE cellulose column in 0.001 M phosphate buffer at pH 6.4 and fractions were collected following increases in the buffer concentration to 0.01 M and 0.3 M. The fractions were dialyzed and lyophilized and tested for cholera toxin content by the Ouchterlony technique, in infant rabbits, and by skin testing in adult rabbits. Essentially all the activity was found in the fraction eluted by 0.3 M buffer. The results indicate that it is feasible to achieve a further degree of purification of cholera toxin by ion exchange chromatography.

Sephadex purified cholera toxin has been analyzed by the sucrose density gradient centrifugation technique. Fractions collected after sucrose gradient centrifugation (10–40 % at 100,000 g for 18 hours) were tested for cholera toxin by the Ouchterlony agar gel double diffusion immune precipitation technique with specific anticholera toxin serum. The cholera toxin antigen was found primarily in fractions 6 and 7 indicating that it has a sedimentation coefficient approximating 6–7 S.

Additionally, an effort is being made to obtain disc electrophoretically pure cholera toxin by the technique of preparative disc electrophoresis. Preliminary attempts to separate serum proteins by this method have suggested the feasibility of its application to the ultimate purification of cholera toxin. When, and if, pure cholera toxin becomes available it should be possible to determine its precise chemical composition and gain further insight into its mode of action.

3. Cholera toxin elaboration in vivo.

In order to learn more regarding the elaboration of cholera toxin in vivo and its relation to the pathogenesis of experimental cholera, a series of experiments was designed to determine if, when, and in what amount, cholera toxin was produced in V. cholerae 569 B infected ligated ileal loops of adult rabbits, and to compare the response to infection with that following inoculation of purified cholera toxin.

Initially, a "standard curve" of the response of isolated loops to varying doses of cholera toxin, in terms of milliliters of fluid elaborated per centimeter of intestine, was constructed for comparison and for use as an additional bioassay of cholera toxin. This curve is similar to that described by Burrows and Mustekis (J. Inf. Dis. 116: 183, 1966) for cholera "whole cell lysate" with the exception that purified cholera toxin is approximately 200 times more active in this model. The time sequence of outpouring of fluid in response to cholera toxin was also studied. From the results, it appears that fluid accumulation begins to appear at about 2 hours, the earliest time when an increase in vascular permeability was detectable in the infant rabbit model, and then proceeds at a rate and to a maximum volume which is determined by the amount of cholera toxin injected initially.

Table I summarizes the data obtained in 88 Benadryl treated and 65 control infant rabbits fed cholera. Three observations were made over an experimental period of 21 hours: death, occurrence of over diarrhea, and the accumulation of intrainstestinal fluid. There were only slight differences between the two groups in the incidence of diarrhea and in the number of animals with autopsy evidence of fluid leakage into the small intestine. However the mortality rate was greater in the untreated animals than in the Benadryl treated group. Increased survival in the latter group may be a result of slower intestinal transit time secondary to drug (see below), allowing re-absorption of fluid in the lower bower and thereby less acute fluid loss. More potent and more specific anti-histaminic drugs should be tested.

Table I

Cholera (ug)	No Benadryl						Benadryl — treated*					
	Death		Diarrhea		Excessive fluid at autopsy		Death		+ Diarrhea		Excessive fluid at autopsy	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
50	17/24	71	22/24	92	23/24	96	16/41	39	34/41	83	38/41	93
25	9/12	75	10/12	83	10/12	83	8/12	67	11/12	92	12/12	100
10	10/24	42	21/24	88	23/24	96	10/30	33	18/30	60	23/30	76
4	0/5	0	3/5	60	4/5	80	0/5	0	1/5	20	1/5	20
All doses	36/65	54	56/65	86	60/65	92	34/88	39	64/88	73	74/88	84

* Animals were given 1 mg of benadryl I.P. 1/2 before, simultaneously, and 1/2 h after feeding of cholera.

Determination of G-I Transit time in normal and benadryl-treated infant rabbits. Carmine red dye, a non absorbable marker, was used as an indicator of transit time. 5 mg of dye was added to 1 ml of phosphate buffer and fed to infant rabbits. One group of animals was given 1 mg of benadryl intraperitoneally twice, 1/2 h before and after feeding of dye, another group was fed dye only. All animals were put in separate partitions of a cage and observed for the appearance of red stool every 15 (and later on 30) minutes. The results are shown below.

Time (h)	Number and percentage of animals excreted dye			
	Control		Benadryl — treated	
	No.	%	No.	%
at-6	0	0	0	0
6-7	3	10.3	0	0
8-9	5	17.2	1	3
11-12	9	31.0	3	10.8
13-20	20	69.0	13	46.8
21-22	25	86.5	16	57.6
at-24	28	96.6	23	82.8
Total No.	29	100.0	28	100.0

Benadryl inhibits intestinal transit time in infant rabbits in a dose of 10 mg per kg body weight. This non specific effect may be the mechanism whereby Benadryl appears to enhance survival in experimental cholera.

7. Production of intestinal immunity by parenteral vaccination with cholera

Adult rabbits were inoculated simultaneously at multiple sites (subcutaneous, intramuscular, intra-peritoneal and in foot pads) with 5 mg of cholera in complete Freund's adjuvant. Three weeks later, the animals were bled for antibody determinations. At that time cholera was assayed in them by intradermal inoculation and by the loop technique described above. In addition, one loop was inoculated with 10^8 vibrios of strain 569 B. The same procedures were performed simultaneously in control animals.

In the intradermal tests, the smallest doses of cholera that produced positive reaction in the control group of animals were 0.005-0.01 ug, while in the immunized group they were 1.25-2.5 ug, 250 times higher. The responses to intraintestinal challenge are summarized in Table II. The results demonstrate conclusively that under these conditions it is possible to produce relatively strong immunity both to cholera, intradermally and intra-intestinally, as well as to the intra-intestinal inoculation of a rather massive challenge of cholera vibrios. Serum from each of the immunized animals, except #5, was demonstrated to contain precipitating antibody against cholera and to neutralize its cholera effect when serum and cholera were mixed prior to feeding infant rabbits. Serum from rabbit #5 had a lower content of cholera antibody and did not completely neutralize cholera fed to infant rabbits. When pooled together and given intraperitoneally to infant rabbits 18 hours prior to oral cholera, these sera offered some protection, although not absolute, against experimental cholera.

Table II
Effect of Parenteral Immunization* with Cholera on
Resistance to Intestinal and Intradermal Challenge

Challenge	Control Rabbits				Immunized Rabbits					
	1	2	3	4	5	6	7	8	9	10
Intra-intestinal 10^8 <i>V. cholerae</i> Cholera	3.3**	7.0	5.8	6.7	2.8	0	2.8	0	0	0
50 ug	—	—	—	—	2.6	0	1.0	5.3	0	0
10 ug	5.1	7.0	5.6	6.6	0	0	2.3	0	0	0
2 ug	0	0.8	4.0	5.0	0	0	0	0	0	0
0.4 ug	0	0	0	4.0	2.5	0	0	0	0	0
0.08 ug	0	0	0	0	—	—	—	—	—	—
Intradermal Cholera	0.005#	0.01	0.02	0.005	1.25	2.5	> 10.0	2.5	1.25	5.0

* Immunized rabbits were inoculated with 5 mg of cholera in Freund's complete adjuvant in multiple sites 3 weeks prior to challenge.

** ml of fluid per inch of intestinal loop, 18 hours after challenge.

Smallest dose of cholera producing+reaction, 18 hours after inoculation. Rabbits 9 and 10 were pre-tested, prior to immunization. The minimal skin reactive doses at that time were 0.01 and 0.15 ug, respectively.

8. Production of experimental cholera in Thiry-Vella loops in adult rabbits.

Preliminary experiments have suggested the feasibility of producing experimental cholera in modified Thiry-Vella fistulas in adult rabbits. Open lengths of ileum, ranging from 15 to 25 cm, were isolated, with blood supply intact, and anchored at both ends to holes in the abdominal wall. The stomata could be occluded with Foley catheters to enable administration of cholera toxin and collection of intestinal fluids. The first two animals were administered 50 µg of cholera toxin, in 5 ml, in the isolated loops. After 1 hour the loops were drained and fluids were collected overnight. During this period approximately 40 ml of "rice water" fluid was excreted. Studies on the electrolyte composition of the fluids are in progress. This model offers great possibilities for controlled studies on the development of immunity and on electrolyte balance.

9. Cholera toxin in the canine model

On the occasion of the NIH Cholera Advisory Committee Workshop on Cholera which was held at Johns Hopkins University School of Medicine, December 6-8, the coordinator, in collaboration with Dr. C.C.J. Carpenter and Dr. Bradley Sack, administered purified cholera toxin to a mongrel dog. The dog, which weighed 14 kilograms, was given 80 mg of cholera toxin by intraduodenal catheter. Fluid produced in the intestinal tract was drained by means of a catheter placed in the terminal ileum. Over a 35 hour period following administration of cholera toxin, the dog lost over 1 liter of clear, rice-water, non-hemorrhagic fluid (from the intestinal drain and in vomitus) confirming the fact that the cholera toxin preparation contains the toxin responsible for fluid and electrolyte loss in (canine) cholera. Previous trials, in which amounts of less than 10 mg were given to dogs in the same manner, suggest that the dog is somewhat more refractory to cholera toxin than the infant rabbit or man.

10. An attempt at improving conventional cholera vaccines

Almost universally, current conventional cholera vaccines are composed of killed vibrios derived from the (U.S.) NIH Inaba and Ogawa serotype reference strains although there is no evidence that these strains are superior to any others. The results of the recent field trials of cholera vaccines have indicated that some presently available vaccines confer some limited degree of immunity against cholera for a short duration. Obviously some improvement is needed. Accordingly, it was decided to attempt to improve conventional vaccines by selection of strains of higher antigenicity.

A test was devised to evaluate the antigenicity of various strains of cholera vibrios in the rabbit. The test was based on the observation that the agglutinin response in rabbits inoculated with a single vaccine dose is directly proportional to the amount of vaccine used. Accordingly, a comparison was made of the agglutinin response of rabbits, 4 per group, inoculated with single doses of monovalent vaccines composed of strains representing each of the kinds of cholera vibrios; El Tor Inaba and Ogawa and classical Inaba and Ogawa. The rabbit sera were each tested first against agglutinating antigens prepared from the NIH reference Inaba and Ogawa strains. On the basis of these results, the "best" and the "worst" strains in each category (of approximately 10 strains per group) were selected and their sera were titrated individually against a battery of agglutinating antigens (8 Inaba and 7 Ogawa types). The geometric mean titers resulting in these assays are abridged in Table III. Comparison of the results with paired "good" and "bad" strains revealed the differences to be statistically significant (95% confidence) in all but the comparison between strains *V. cholerae* 569 B and IDH 59. Based upon these results, two quadrivalent vaccines, a "good" one and a "bad" one, were composed of the odd and even numbered strains (Table III), respectively. These have passed rigid animal and laboratory tests for sterility, safety and toxicity. The quadrivalent vaccines were compared by a dose-response antigenicity assay. The results of this test, in which graded single doses of the vaccines were administered to groups of rabbits which were bled for agglutinating and vibriocidal.

Table III

Comparison of geometric mean agglutinin titers* elicited
by "good" and "bad" vaccine strains

Strain Number	Designation	Geometric Mean Titer**95% C.L.	
1	<u>V. chol.</u> Inaba 569 B	760.8	475 - 1219
2	" " IDH 59	603.8	369 - 784
3	<u>El Tor Inaba</u> BRL 7738	1070.0	810 - 1430
4	" " HP-51-1	356.7	270 - 471
5	<u>V. chol.</u> Ogawa 12RX1	1312.0	1047 - 1644
6	" " VN Dalat	320.0	249 - 412
7	<u>El Tor Ogawa</u> VN 258	689.5	560 - 848
8	" " Teheran 816-0	362.1	256 - 489

* Based upon titrations of 4 sera per strain against a battery of agglutinating antigens (8 Inaba types and 7 Ogawa types)

** Homologous antigenic type titers only.

antibody determinations after two weeks, indicated that the test had all the attributes of a valid bioassay and that the response to the two vaccines was significantly different* by a factor which represents their relative potency in this test. With regard to the Inaba agglutinin response, Vaccine A is 13.48 times more potent than Vaccine B (95% C.L.=5.415 and 33.59). With regard to the Ogawa response, Vaccine A is 8.990 times more potent (95% C.L.=4.197 and 23.15).

The two vaccines were assayed in the standard mouse potency test by Dr. John Feeley, Division of Biologics Standards, National Institutes of Health. Interestingly, in those assays, the two vaccines did not differ significantly and were found to be equivalent to reference standard potency preparations.

It is hoped to project these studies to small groups of human volunteers who will be given single doses of diluted vaccines to determine if the rabbit assay predicts the human serological response.

* We are indebted to Col. S. Vivona, Director, US Army Medical Component, SEATO Medical Research Laboratory for his assistance in the statistical interpretation of these data.

Summary—

1. Cholera toxin is produced in a completely defined medium supplemented with 16 L-amino acids. Purified cholera toxin has been isolated from crude cholera culture filtrates produced in rather large volumes at WRAIR. A non-cholera toxin elaborating strain did not inactivate pre-formed cholera toxin.
2. DEAE cellulose may be useful in further purification of cholera toxin. Cholera toxin has been identified by sucrose density gradient centrifugation as having a sedimentation coefficient approximating 6.7 S. The technique of preparative disc electrophoresis is being investigated for possible application to the ultimate purification of cholera toxin.
3. Cholera toxin is elaborated in vivo in ligated loops in adult rabbits by some strains of cholera vibrios during the phase of their logarithmic increase in numbers during infection. It can be assayed by skin reactivity and by precipitation with antibody in Ouchterlony tests. Some strains which fail to produce immunologically detectable levels of cholera toxin in vivo may produce the small amounts which are required to initiate the positive loop.
4. Histological observations on the development of the positive loop in response to cholera toxin indicate that the necrosis and epithelial detachment are a consequence of ischemia due to increased intraluminal pressure.
5. Cholera toxin has been shown to alter vascular permeability in the small bowel of infant rabbits. The vessels involved are primarily the venules.
6. Some anti-histaminics may exert a slight non-specific apparent protective effect on experimental cholera in infant rabbits.
7. Immunization with purified cholera toxin in Freund's adjuvant gave rise to a strong degree of intestinal immunity to challenge with cholera toxin or with live vibrios in adult rabbits.
8. The Thiry-Vella fistula, in adult rabbits, offers great possibilities for controlled studies on the development of immunity and electrolyte balance in cholera.
9. Cholera toxin also causes a cholera-like syndrome in the canine model.
10. A new assay has been developed for selecting highly antigenic strains for inclusion in conventional cholera vaccines. Vaccines composed on the basis of this test were found to differ significantly in their relative potencies in the antigenicity test but not in the mouse protection test. It is hoped to project these studies to small groups of human volunteers to determine whether this laboratory test predicts response in man.

Publications —

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