

STUDY REPORT

Title: Pathogenesis and Immunology of Cholera

Principal Investigators: Pongsom Atthasampunna, M.D.
Richard A. Finkelstein, Ph.D.
Bernhardt W. Langer, Jr., Ph.D.

Associate Investigators: Peter K. Iber, MAJ, MSC
David M. Robinson, LTC, MC

Assistant Investigators: Panyasri Benjadol, M.S.
Yael Friedlander, MSG
Duangduen Jiampermphoon, B.S.
Chanphen Laongkeo, B.S.
Pavanee Prayongratna, R.N.
Pantipa Sinrachatanand, B.S.

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

Description Efforts during this year have been concerned with:

1. Production of cholera in a simple completely defined medium.
2. Purification of cholera by preparative disc electrophoresis.
3. Production of antibodies and pathological changes in rabbits immunized with purified cholera preparations.
4. Experimental cholera in the Thiry-Vella loop of adult rabbits.
5. Intragastric administration of cholera to rhesus monkeys.
6. Cholera in the Thiry-Vella loop of sheep.

Progress

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

Efforts toward developing a simple, chemically defined medium for the production of cholera have continued. Cholera production has been obtained in a sucrose base supplemented with 16 amino acids in lieu of the casamino acid supplement normally used. Sodium lactate was substituted for sucrose as a carbon source with satisfactory yields. This prevented development of acidity in improperly aerated media and may be useful in batch production, as practiced at Walter Reed Army Institute of Research, Washington, D.C. (to date these batch productions have been less than satisfactory). Attempts to simplify the lactate medium with amino acid supplement have given conflicting results. A comparison was made of the effect of dialysis in distilled water as opposed to dialysis in 0.02 M NH_4HCO_3 , a volatile buffer. Yields of cholera and cholera activity were found to be similar under both conditions. However, we now use NH_4HCO_3 as buffer/eluent in Sephadex columns instead of phosphate buffer to save one dialysis step.

2. Purification of cholera by preparative disc electrophoresis

After preliminary analysis by analytical disc electrophoresis, 175 mg of regular purified cholera (cholera which had been collected and concentrated following passage of crude, dialyzed and lyphi-

lized culture filtrate through Sephadex G-200) were separated by preparative disc electrophoresis using the Buchler apparatus. According to the preliminary tests, the cholera antigen containing region (as determined by Ouchterlony agar gel double diffusion) of the analytical disc contained between 16 and 20% of the total protein nitrogen. Fractions from the preparative run which contained antigen activity detected in the Ouchterlony test were pooled, dialyzed and lyophilized. The yield was 18.5 mg of an off-white powder. Analytical disc electrophoresis of this material indicated that approximately 55% was in the cholera antigen region. However, paradoxically, according to data to date, this did not result in a concomitant increase in the specific activity of the product. Results are summarized in Table 1.

3. Production of antibodies and pathological changes in rabbits immunized with purified cholera preparations

Adult rabbits were inoculated simultaneously at multiple sites—intramuscular, subcutaneous, intraperitoneal and in foot pads—with 5 mg of regular purified cholera (No. 1, 3, TV19 and TV21), and 5 mg of disc-electrophoretic-purified cholera (No. 2 and 4*) in Freund's adjuvant. The animals were bled 3, 5 and 8 weeks later to obtain sera for antibody determinations. Skin tests were performed on the day of immunization and 3 weeks later.

Results are summarized in Tables 2 and 3. The disc-electrophoretic-purified cholera did not elicit agglutinating antibody or substantial rise of vibriocidal antibody (both are anti-bacterial antibodies). The regular purified cholera elicited both anti-cholera and antibacterial antibodies.

Rabbit No. 2 died 3 weeks after immunization. Post-mortem examination revealed several small areas of white fatty infiltration of two lobes of the liver and small area of hemorrhage of the right lung. Sections of the liver showed large granulomas composed of histiocytes containing an oily or fatty material. The lung showed similar but smaller granulomas. These granulomas were thought to be secondary to Freund's adjuvant. In addition, the arterioles, small arteries, and venules of the lungs were greatly thickened and surrounded by eosinophils. These changes are consistent with serum sickness.

Rabbits No. 1, 3, TV 19 and TV 21 were sacrificed six months after immunization. Lungs, liver, kidneys and spleen of each animal were removed for histological examination. No granulomatous lesions in the livers or lungs were found. The walls of large and medium size arteries of the lungs of all animals were greatly thickened and edematous, and often infiltrated by polymorphonuclear leucocytes; findings which are consistent with serum sickness. The spleens were unremarkable except for questionably thickened penicillary arteries in two animals. There was increased proteinaceous material in glomeruli of the kidneys of two rabbits, a finding suggestive of proteinuria.

4. Experimental cholera in the Thiry-Vella loop of adult rabbits

Studies designed to explore the feasibility of using Thiry-Vella loops in adult rabbits as an experimental cholera system for studying pathogenesis and intestinal immunity have continued. Fourteen more Thiry-Vella fistula animals were operated. Two weeks after operation, both ends of the loops were occluded with Foley catheters and loop secretion (control) was collected. Challenge with cholera was done on the following day. Five ml of PSS containing 50 mcg of cholera were introduced into the loop. After one hour, the loop was emptied, flushed with PSS and fluid accumulations were collected hourly for 8 hours. Aliquots were analyzed for electrolyte content. Skin tests with the same cholera preparation were performed just before challenge and results were recorded the following day. The second cholera challenge was done two weeks later and was followed, in another two weeks, by live vibrio challenge. For *V. cholerae* challenge, a volume of 5 ml of 0.1% peptone in PSS containing 10^8 *V. cholerae* 569B Inaba was introduced into the loop.

Only six animals were usable for 2 challenges with cholera and four with cholera and live vibrios. The results, which are summarized briefly in Table 4, indicate that isolated segments of ileum, not in contact with bile and pancreatic secretions, increased secretion when challenged with cholera or with live vibrios. One hour of incubation was sufficient to produce an outpouring of fluid. Fluid production reached its peak in the fourth and fifth hour after cholera administration and remained

*Rabbit No. 4 died one week after immunization and was excluded from the study.

increased during the first 8 hours. By 24 hours, fluid production was only slightly more than normal. Changes, if any, in the electrolyte composition were not sufficiently consistent to permit any conclusions on this aspect.

The stomata of the loops became smaller after each experiment. In many cases the intestine extruded, was strangled and required surgical repair. On post-mortem, loops were found to be adhesive to the abdominal wall, kinked, and much shorter than when originally made. However, histological observations revealed essentially normal villi in these loops. The progressive shortening of the intestines would make it difficult to obtain consistent results in the same animal in prolonged studies.

5. Cholera in rhesus monkeys

While visiting the Pakistan-SEATO Cholera Research Laboratory in Dacca, East Pakistan, the principal investigator (P.A.), in collaboration with Dr. M.M. Rahaman, infused 100 ml of crude Syncase cholera toxin via gastric tube into the stomach of each of two rhesus monkeys pre-fed with NaHCO_3 . One monkey, weighing 2.4 kgs, produced 270 ml of liquid stool over a six hour period beginning two hours after infusion. The electrolyte contents of the stool approximated that found in the stools of cholera patients. The other monkey did not respond. Dr. M.M. Rahaman planned to repeat this experiment.

6. Cholera in the Thiry-Vella loop of sheep

During the same visit, the principal investigator (P.A.), supplied Dr. Nalin with some purified cholera toxin (lot July 67). He administered 200 mcg into each Thiry-Vella loop of two sheep. There was no remarkable response.

Summary

1. Attempts to define the amino acid requirement of V. cholerae inaba 569B essential for production of cholera toxin gave conflicting results. Sucrose could be substituted by sodium lactate as a carbon source in the culture medium.

2. The specific activity (choleraogenicity) of cholera toxin purified by means of preparative disc electrophoresis did not concomitantly increase with its purity.

3. Cholera toxin purified by our regular process elicited antibacterial and the expected anti-cholera toxin antibodies but cholera toxin purified by disc electrophoresis elicited only anti-cholera toxin antibodies. The antibody level was sustained for at least 8 weeks in all animals. Post-mortem examination of the immunized rabbits revealed pathology in the lungs consistent with serum sickness.

4. Administration of cholera toxin or live vibrios increased secretion in isolated Thiry-Vella ileal loops of rabbits. The stricture of the stomata and the progressive shortening of the loops precludes the usefulness of this experimental model.

5. One of two rhesus monkeys had diarrhea when fed cholera toxin.

6. Sheep Thiry-Vella loops did not respond to cholera toxin.