SEATO MEDICAL RESEARCH STUDY ON CHOLERA

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Title: 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.

Description — Efforts during this year have been directed primarily to the isolation and indentification of choleragen and to the determination of its mode of action. Choleragen is an antigenic moiety which is elaborated *in vitro* by certain strains of cholera vibrios. It causes experimental cholera when administered *per os* into the stomachs of infant rabbits and it has been shown to cause cholera experimentally in man when administered into the lumen of the small intestines. Although two components, Procholeragen A and Procholeragen B, were formerly considered to be essential, it is now recognized that Procholeragen B is simply a buffer which serves to protect Procholeragen A against destruction under the acid conditions in the stomach. Hence, these now unnecessary terms should be discarded in favor of the single descriptive term, choleragen, which is identical with the fraction formerly regarded as Procholeragen A. By use of the technique of disc immunoelectrophoresis with polyacrylamide gel, choleragen has been identified as a protein which migrates electrophoretically in this porcedure as does human 7 S gamma globulin. Choleragen which has been passed through the molecular sieve, Sephadex, is still not pure but contains a number of other components detectable by disc electrophoresis. Among these components is cholera mucinase which, our results have led us to conclude, plays no role in the biological activities of choleragen.

The choleragen moiety appears to be responsible for the diarrhea of experimental cholera in infant rabbits. On intradermal inoculation in minute amounts, it causes a delayed but sustained local reaction in the skin of rabbits which is characterized by an erythematous, edematous induration which "fixes" intravenous pontamine sky blue dye. Choleragen in small doses also causes outpouring of fluid into ligated loops of small intestines of adult rabbits. Based upon these and other observations, it is proposed that an increase in vascular permeability brought about by the action of choleragen, or choleragen-like factors, elaborated by cholera vibrios in the small intestines is a major factor in the pathogenesis of cholera.

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In an attempt to develop a primate model which might reproduce in the laboratory the manifestations of cholera as it occurs in man, choleragen was administered at laparotomy into the small bowel of three gibbons. Although the dosage used was many times that which would be expected to evoke 20 liter diarrheas in humans, the gibbons manifested only a few loose movements and no other untoward effects. The results indicate that the gibbon is refractory to choleragen and imply that further studies may safely be conducted in human volunteers.

In addition, an epidemic of cholera, caused by Inaba serotype El Tor vibrios, in Korat, Thailand is described. It is believed that efforts at control, dependent primarily on rapid recognition and isolation of inapparent infections in the households of cases, were effective in limiting further spread of the epidemic.

Progress -

1. Identification of Procholeragen B as buffer

We had long been puzzled by the apparent requirement of two camponents for the production of experimental cholera in infant rabbits. Following observations that conditions of acid pH were detrimental to Procholeragen A activity (even with subsequent addition of Procholeragen B), it was considered that Procholeragen A might be destroyed in the stomach of infant rabbits. Subsequent experiments revealed that Procholeragen A was effective, by itself, when instilled into the small intestines of infant rabbits and was not augmented, in producing the skin reaction in adult rabbits, by the addition of Procholeragen B, Several buffer sytstems successfully substituted for Procholeragen B in allowing the activity of Procholeragen A when administered per os into stomach of infant rabbits providing that the buffering was on the alkaline side. Present procedure, which calls for admixture of purified Procholeragen A (now choleragen) wiih an equal volume of phosphate buffer (a mixture of 2 % each of dibasic sodium and potassium phosphates) prior to feeding infant rabbits, is almost invariably successful in producing choleraic diarrhea and/or excessive fluid in the intestinal tract at sacrifice. A pool of several lots of Sephadex purified choleragen has been prepared which is effective in infant rabbits administered 4.0 ug in phosphate buffer.

2. Lack of effect of choleragen on electrolyte transport

Choleragen has now been tested by two groups of investigators for its ability to affect electrolyte transport. The NAMRU-2 group in Taipei, through the cooperation of Drs. R.A. Phillips and E. Neptune, evaluated coded samples of crude Syncase choleragen and a control preparation for their ability to inhibit the "sodium pump" of frog skin membranes. Both preparations, active choleragen and control, gave a modest inhibition of the short circuited current, ranging between 10 and 18% in repeated tests, Which was reversible by washing the frog skin. After heat treatment, both preparations showed approximately the same degree of inhibition. Thus, the frog skin assay could not differentiate between a preparation of proven Choleragenicity (which we have shown to be heat labile) and an inactive control. A Sample of highly active purified choleragen has been tested, by Dr. Michael Field and Dr. Stanley Schultz of the Biophysical Laboratory at Harvard, on isolated segments of rabbit ileum. As a control, heated choleragen was used. "In no instance was there any difference in electric potential or short circuit current between the ileum treated with active and inactivated choleragen. The potentials and currents obtained were in the range to be expected from normal preparations of ileum. This proved to be the case even when ileum was exposed to 200 pg/ml of the choleragen for more than 1 hour."

Thus, current opinion notwithstanding, if choleragen is indeed the diarrhea-provoking moiety of the cholera vibrio, then "sodium pump inhibition" as measured in these systems bears no relation to choleragenicity.

3. Skin reactivity af choleragen

It was previously observed (Finkelstein and Barbaro, 1962, unpublished observations) that crued choleragenic filtrates and sonic lysates of heavy suspensions of living cholera vibrios caused skin reactions which were manifest as a delayed but sustained type of reaction which reached near maximum proportions of erythema, edema and induration at the site of inoculation after 4 hours and persisted for at least 48 hours. Craig has found this heat labile activity in stools of cholera patients and in V. cholerae culture filtrates. Accordingly, it was of obvious importance to determine whether this lesion was caused by purified choleragen. Initial experiments revealed that fractions obtained from Sephadex columns during the purification of choleragen had activity only when choleragen, detectable by Ouchterlony assay with specific antibody and/or by feeding infant rabbits, was present in the fraction. Purified choleragen elicits this skin reaction when amounts of the order of 0.075 ug are injected intradermally in rabbits. This purified choleragen is estimated to be only 25-35% pure (see below). Thus choleragen ranks among the most active of the vasoactive substances such as histamine and bradykinin. Intravenously injected protein-binding dyes such as Evans Blue or pontamine sky blue are "fixed" at the site of the reaction thus indicating that it is associated with an alteration of vascular permeability. The sequential development of this lesion has been studied extensively by histologic techniques and by observation of the migration of intravenously administered India Ink. Adult rabbits were inoculated intradermally with 10 ug of purified choleragen at intervals prior to sacrifice. One hour before sacrifice the animals were administered India Ink intravenously. Immediately after sacrifice, the skin was removed and previously identified areas of inoculation were processed for histological observations. The initial changes, already manifest to some degree 1 hour after inoculation, consisted of endothelial swelling, congestion and dilation of capillaries, peri-capillary edema and infiltration around capillaries and small venules of small numbers of heterophiles. Carbon particles could be identified in the vascular endothelium at that time. Shortly after this, edema was increased, fibrin was apparent in the interstitial tissue, perivascular hemorrhage and diapedesis of red cells were prominent and considerable carbon was present free in the interstitial tissue. By 4 hours, an intense heterophilic vasculitis was present and a few mononuclear cells appeared in the infiltrate. After 24 hours, the infiltration of heterophils had become diffuse and less prominently perivascular. Macrophages were present in increasing numbers. By two days, the infiltrate was composed of over 80% mononuclear cells. The tissue was very edematous and there were large pools of fibrin between widely separated collagen fibers. With smaller doses of choleragen, these changes were less marked and none of these alterations were evident at the site of control (saline) inoculations at any time interval. The changes observed were consistent with a profound alteration of vascular permeability. This lesion may be useful in interpreting some of the changes which may occur in the intestines in cholera although one must bear in mind the functional and structural differences between skin and intestines.

4. Attempts to define vascular leakage in the intestine

Although there is histologic evidence of proteinaceous edema in the lamina propria of the villi in the natural disease of man as well as in the experimental disease of the infant rabbits, it would be desirable to demonstrate by other techniques that vascular leakage does indeed occur. Accordingly, attempts were made to quantitate the reaction by performing determinations of pontamine sky blue (intravenously administered) in the intestinal tissue of choleraic and control infant rabbits in relation to the hemoglobin content (to enable evaluation of the effect of congestion). Unfortunately these experiments were unsuccessful for technical reasons. We have now attempted to employ the technique of "vascular labeling" devised by Majno and Palade. This technique involves intravenous injection of a colloidal suspension of carbon (India Ink) in a dose which is completely cleared by the reticuloendothelial system of normal animals. Vessels with altered permeability leak carbon particles between endothelial cells where they are trapped by the basement membrane. The vessels, in cleared tissue, are then viewed under the dissecting microscope. The results of application of this technique with infant rabbit intestinal preparations

have not revealed consistent changes in the vilus vessels of choleraic animals and further study is in progress using smaller colloidal particles. The preliminary results of electron micrographic study (performed in collaboration with Smith and Metzger at the 406th Medical Laboratory) of intestinal tissue from choleraic and normal infant rabbits administered ferritin have indicated that ferritin particles escape from intestinal vessels in choleraic but not in normal animals. Dr. Guido Manno, of the Department of Pathology at Harverd, who is studying the effects of choleragen on skin and muscle, has indicated that choleragen produces a strong delayed histamine-type response in rat skin and cremaster muscle characterized by an increase of venular permeability.

5. Characterization of choleragen by disc immunoelectrophoresis

Samples of purified choleragen were separated electrophoretically in polyacrylamide gel columns which were then imbedded in agar in Petri dishes and allowed to react with choleragen antiserum placed in troughs in the agar parallel to the long axis of the electrophoresed gel. A single precipitin band formed which corresponded with a dense protein-staining component of relatively slow mobility, thus identifying choleragen antigen as a protein with mobility and size similar to human 7 S gamma globulin. Other protein components were also present. As many as cleven peaks could be detected by scanning with a microdensitometer. However, none of the components reacted with stains for glycoprotein or lipoprotein. The area of antigenic activity contained from 27 to 38% of the total protein in different preparations.

6. Mucinase activity of choleragen preparations

The mucinase of cholera vibrios, which was shown by Burnet and Stone to associated with desquamation of intestinal epithelium in vitro, was, for some time, considered to bo involved in the pathogenesis of cholera. Following the observations of Gangarosa et al. that the intestinal epithelium was intact in cholera, interest in the mucinase ceased abrupty. However, it should be recalled that Burnet and Stone cautioned that the desquamation observed in vitro was a highly artificial reaction completely dependent on autolytic changes. Therefore it was of interest to determine the mucinase activity of our choleragen preparations. Preliminary experiments revealed that crude choleragenic filtrates contained mucinase activity (as assayed with ovomucin) and that, as the choleragenic activity was purified and concentrated, the mucinase activity increased. Furthermore, the mucinase activity was neutralized by antiserum against choleragen and was not affected by antisera against living vibrios which have no choleragen neutralizing activity. Cholera mucinase, prepared by modification of published techniques, was choleragenic, but not as active in this regard as purified choleragen. Approximately 100 ug were required to produce choleraic manifestations in infant rabbits whereas only 4.0 ug of purified choleragen were needed. Similarly, the "mucinase" was less mucinolytic than purified choleragen; 62.5 ug constituting a mucinase unit while only 4.0 ug of choleragen were required for the same effect. Although these results suggested the identity of choleragen and mucinase, a clear cut separation between the two activities was obtained following ammonium sulfate fractionation. Purified choleragen was treated by either 1/2 or 2/3 saturation with $(NH_4)_2SO_4$ and the supernatant and precipitate fractions collected, dialyzed and lyophilized. The fractions were then tested for choleragenicity and mucinase as well activity as for antigen precipitating with choleragen antibody in Ouchterlony tests. In addition, the fractions were titrated for skin reactivity by intradermal inoculation of adult rabbits. Following 2/3 saturation with $(NH_4)_2SO_4$, the precipitate contained most of the mucinase activity; the supernatant being 250 times less active. However the choleragen was nearly equally distributed in the two fractions as demonstrated by both choleragenicity tests and Ouchterlony assays. Following 1/2 saturation, the supernate was approximately 20-fold less active as mucinase, but was approximately 5 times more active as a choleragen. The skin reactivity was also associated with the choleragen moiety rather than the mucinase. It is evident that the biological activities of purified choleragen are dependent on the choleragen moiety and not the mucinase. Syncase filtrates (lyophilized

and concentrated 10-fold) of 24 additional strains were also tested for mucinase activity, choleragenicity, skin reactivity, and for antigen reacting with choleragen antibody in the Ouchterlony procedure. Although a wide range of mucinase titers was obtained, most being as active or more active than our routine strain only one filtrate was choleragenic. This, interestingly, was from an Ogawa serotype El Tor vibrio isolated in Viet Nam. Ist choleragen was antigenically identical with that of our routine strain, V. cholerae 569 B Inaba. Although most of the filtrates exhibited some activity in rabbit skin at dilutions through 1:10, the choleragenic filtrate was active at 1:10,000. Only the choleragenic filtrate gave a reaction in the Ouchterlony test. These results further emphasize (1) the association of choleragenicity with the skin reaction, (2) the distinction between mucinase and choleragen, and (3) the specificity of the immunologic test for choleragen. Most af the strains tested had previously or recently been found to cause cholera experimentally in infant rabbits inoculated intraintestinally with 10^4 living vibrios. The reason why more strains do not elaborate choleragen in vitro remains obscure. Although it is possible that the diarrhea of cholera may be brought about by more than one mechanism, it seems more likely that the conditions which we have selected for the production of choleragen in vitro are unsuitable for the majority of potentially capable strains. It is possible that those strains which do elaborate substantial amounts of choleragen in vitro are aberrant strains in the same sense as are strains of microorganisms used in industry for the production of commercial products, which elaborate large amounts of a normally intermediate product because they lack the capacity to utilize it further under the conditions of the industrial fermentation. These results also raise the question of the nature of the low titer skin reactive substance present in most of the filtrates. It remains to be determined whether this is choleragen in small amounts or a different factor.

7. Choleragen in gibbons

In an attempt to find another experimental model which can be used in further studies of the pathogenesis and immunology of cholera, choleragen was administered, at laparotomy, in 10 mg doses, into the small intestines of three gibbons. Although this dose is approximately three times that which might be expected to produce a 20 liter diarrhea in man, the gibbons each responded with only one or a few loose movements. Electrolyte determinations performed on serum samples taken the mornming after choleragen administration failed to reveal any significant changes which might be construed as reflecting the physiologic disturbance found in cholera in man. One gibbon exhibited a slight loss of appetite but no other effects were noted. The results indicate that the gibbon is refractory to choleragen and suggest that further studies may be conducted in man with an additional degree of safety.

8. Choleragen in ligated loops of adult rabbit intestine

Preliminary experiments have revealed that choleragen is extremely effective in provoking outpouring of fluid into the lumen of isolated loops of adult rabbit small intestines. Although there is some rabbit to rabbit variation, doses as small as 0.4 ug have resulted in positive resutts. The response appears to be dose related.

9. The "Samlor" cholera outbreak in Korat, Thailand

On 7 January 1966, the Thai Department of Health informed us that a few cases of cholera had been reported in Korat, Thailand. Our offer of assistance was accepted and a team departed the same day for Korat. The team consisted of a bacteriologist experienced with rapid techniques for isolation of cholera vibrios, a physician cholera research worker, an epidemiologist, two field nurses, a technician and sufficient supplies to enable the processing of 400 bacteriological specimens without further delay. The same night, and through succeeding days and nights, the team visited the house of new cases and performed rectal swabs on all household contacts. Using the oblique light technique of Finkelstein and Gomez, it was possible to provide local health officials with results within 12 hours of receipt of the specimen.

The philosophy, based on previous experience that a high proportion of household contacts were bacteriologically positive, was that if the inapparent infections could be detected rapidly and prevented from disseminating the vibrios further, the epidemic might be contained in Korat or even aborted. In addition, the team furnished bacteriological support at the Korat Provincial Hospital, attempted to conduct a field trial of tetracycline prophylaxis in an outbreak in the Korat Prison, and tried to do a comparative study of the efficacy of sulfa, tetracycline and chloramphenicol in the treatment of cholera cases and carriers. Cantor tubes were "dropped" for the collection of samples from the small intestines of cholera carriers to determine if there were vibrios in the small bowel.

Of 111 household contacts investigated, 9 bacteriologically positive individuals were detected and removed to the Provincial Hospital. Fifty per cent of the twelve households visited had bacteriologically positive individuals revealed by a single sampling. One neighborhood contact was positive among 16 individuals investigated in 5 houses. Three inapparent infections were detected in the prison population. Of these, 2 were food handlers (of the 44 food handlers who were swabbed). Of the 13 bacteriologically positive individualspickedup by this survey, 7 either previously or subsequently had diarrhea. Including theses 7 individuals, who were then regarded as cases there was a total of 31 cases. All were associated with El Tor vibrios of the Inaba serotype. Cholera vibrios were not isolated from 93 additional diarrheal patients admitted during the outbreak. The last cholera patient was admitted, after a six day lapse in cases, on 19 January.

The epidemic was traced from the initial case which occurred in a visitor who had come to Korat from Khonkaen three days before she had symptoms. She lived in a house near the prison where cases occurred quite early in the outbreak. Additional cases occurred in the prison and nearby and then in a cluster of "Samlor houses" not far removed. Samlors are three wheeled bicycle type vehicles which are the major means of transportation in Korat. Some 200 samlor drivers operated out of these samlor houses. Subsequent cases, removed from the original focus, were associated with samlor drivers who came to these samlor houses to pick up their vehicles. Of the 31 confirmed cases, 8 occurred in samlor drivers and 8 in the prison.

The attempted trial of tetracycline prophylaxis in the institutional outbreak was unsuccessful due to the paucity of positive findings following removal of the infected food handlers.

The hospitalized cases presented a wide range of clinical manifestations from mild diarrheas which required little if any treatment to severs cases requiring more than 20 liters of I.V. fluid replacement. The earlier cases (unfortunately the majority) were treated with sulfasuxidine which appeared to have no effect either on the clinical course or on the period of excretion of vibrios. Chloramphenicol appeared to have a beneficial effect in shortening the period of vibrio excretion, but the small numder of patients studied precludes drawing any firm conclusions. Chloramphenicol does merit further testing as a cheaper (and perhaps more effective) substitute for tetracycline.

Small bowel fluids collected from two carriers, who were pospitive rectally at the time of intraluminal sampling, did not contain cholera vibrios. This observation is of some interest since it suggests that one difference between carriers and cases may be the actual location of the vibrios in the intestinal tract.

Summary

Procholeragen B has been identified as buffer which serves only to protect the choleragen (Procholeragen A) against destruction under the acid conditions of the stomach of infant rabbits. Choleragen has been identified as a protein which migrates in disc electrophoresis on polyacrylamide columns as does human 7 S gamma globulin. Choleragen which has been purified by passage through

Sephadex columns is still not pure but contains as many as 11 other protein components recognizable by the sensitive technique of disc electrophoresis. Choleragen antigen constitntes between 27 and 30 % of the nitrogen content of the preparations. Among the other components is cholora mucinase which, it has been shown, is not involved in the biological activities of choleragen. Choleragen does not interfere with electrolyte transport in two in vitro systems, the frog skin membrane and isolated rabbit ileum. In submicrogram levels, choleragen causes an alteration in permeability of the vasculature of rabbit skin characterized by a delayed but sustained erythematous edematous induration at the site of inoculation. Choleragen is also effective in small amounts in causing an outpouring of fluid in loops of rabbit of small intestine in vivo. Based upon these and other considerations, it is proposed that an alteration of villus vascular permeability, brought about by choleragen or choleragen-like factors elaborated by cholera vibrios in the small intestines, is a major factor in the pathogenesis of the diarrhea of cholera. An Ogawa serotype El Tor vibrio has been found to elaborate choleragen in vitro which is antigenically identical with that produced by our routine strain, an Inaba serotype classical vibrio. Gibbons are relatively refractory to intraintestinal inoculation with 10 mg of purified choleragen, a dose which is estimated to be more than 3 times that required to cause a 20 liter diarrhea in man. Aside from a few loose movements, no untoward effects were noted in the 3 gibbons tested. An outbreak of cholera, caused by El Tor vibrios of the Inaba serotype, in Korat, Thailand is described. It is believed that prompt (8-12 hour) identification and isolation of asymptomatic infections contributed materially to the containment of the epidemic. Cholera vibrios could not be isolated from the small intestines of two carriers who were bacteriologically positive by rectal swab at the time of intraluminal sampling.

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