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Leading article – Tropical infection of the gastrointestinal tract and liver series

Cholera and severe toxigenic diarrhoeas

The reintroduction of cholera into South and Central America,[1] and its rapid spread through the region, illustrate the continued susceptibility of most of the world's population to this often lethal disease. This susceptibility is based on attributes of the pathogen, *Vibrio cholerae*, and its bioecology, and on the human host. Host factors include the strength (and weaknesses) of the gastric acid barrier, the faecalisation of the environment leading to contamination of drinking water and food with the pathogen, and the continued lack of education or habit formation sufficient to curtail intestinal contamination and colonisation by *V cholerae*. The host immune response which develops too late to protect the victim and gives variable protection to the survivor and to vaccinees.

Cholera can maintain itself and increase when epidemic strains of *V cholerae* persist longer in the environment and multiply to higher concentrations in water, other beverages or food, notably if chitin is present.[2] The pathogen can pass through the stomach because of the absence of normal acidity or, when acid is present, by attachment to or within a particle. The vehicular particles may be of chitin[2] zoo – or phytoplankton[3] or possibly algae,[3-4] protozoa,[5] cellular debris,[6] parasitic nematodes and their eggs[7] or other substrates offering protection from acidity or other noxious environmental influences, such as heat,[8] cold,[9] and low salinity.[8] *V cholerae's* environmental survival and transmission may be linked to these vehicles.

Vaccine development has consistently produced vaccines giving only a modest protection from disease (at best 50–85%) for periods typically too short (six months – one year and after this period of time, a protection of 0 to 50%) to be of practical significance in all age groups.[10-13] Treatment remains the main area of progess in research, with repeated findings of the success of intravenous and oral replacement of the water and electrolyte losses characterising the disease. This article will attempt at analysing how recent and future research could help to understand and to better control the cholera problem.

The causative vibrio and its bioecology

After the initial hypothesis[14 15] based on analogy with Japanese and American studies of *V parahaemolyticus* coupled with *V cholerae's* then unexplained chitinase production, that an estuarine or other aquatic niche for *V cholerae* might exist in copepods or other chitinaceous fauna, a number of confirmatory studies have been published.[16] Such a niche is firmly established,[3 16] and vibrio adhesion to chitin from such organisms, or ingestion of vibrios in certain foods, can provide safe passage through the gastric acid barrier.[2] Further study will define the comparative rank importance of copepods, free living[3 17] or parasitic on fish,[18] molluscs[19 20] or mammals[21]; isopods,[22] other plankton,[3] crabs[16 23] prawns[9 24] or other organisms, which are chitinaceous or chitinophagous, or parasitised by copepods or other vibrio-carrying organisms, in maintaining and disseminating epidemic *V cholerae* 01 serovar strains in the environment and in human ingesta. The role of birds in transferring *V cholerae* to new aquatic sites has also been suggested.[25]

The recognition that dormant, viable but non-culturable forms of *V cholerae* may persist in acquatic environments is potentially of great significance.[16] Infection of two volunteers challenged with a dormant form of a live gene deletional attenuated vaccine strain of *V cholerae* has been shown,[3] but the comparative importance of wild, dormant forms in cholera pathogenesis and epidemiology, including their ability to cause disease directly in the human host, needs to be determined.

The full range of potential extrahuman hosts of *V cholerae* awaits definition. Findings on *V cholerae* colonisation of intestinal nematodes and attachment to their eggs[7] and multiplication (with lethal effect) in earthworms[26] remain unpursued despite potentially important bioecological implications. Study of the fate of colonised expelled parasitic nematodes, and of earthworms naturally infected by consumption of faecally contaminated soil, after ingestion of infected worms by natural predators and scavengers, could show additional survival or multiplication niches. The possible persistence or multiplication of *V cholerae* in other chitin enriched natural microenvironments, such as in water suitable for ovoposition and larval development by mosquitoes, for example, in crab holes,[27] or in the intestinal tracts of fish harbouring vibrios,[28] some of which maintain ingested chitin residues in their intestines,[29] or in other chitinivorous marine animals, will probably be a useful line of research. There is a need for additional studies of seasonal variations of *V cholerae* 01 counts (culturable and 'non-culturable') in flora,[30] chitinaceous fauna, sediments and water columns of freshwater sources, and the relation of counts to variations in salinity, temperature, and nutrient content[31] of surface and other drinking water sources in endemic areas.[8] Given that one copepod may harbour 10^5 vibrios,[3] $\geq$one infective dose$_{50}$,[32] it is not hard to imagine how small numbers of such aquatic organisms, if imbibed in drinking water, could provide a vehicle for gut contamination, colonisation, and multiplica-

tion leading, with or without prior multiplication in food contaminated by such water, to infection of humans. Ingestion in a chitin vehicle is the mode of infection of ayu fish with *V anguillarum* and offers a possible vehicle (live or killed) for oral vaccines against enteric vibrio infections of humans as for ayu.[33]

The fate of the ingested pathogen, bound or unbound to a copepod or to other microfauna or flora occurring in local drinking water, also revolves around the still underinvestigated phenomenon of tropical hypochlorhydria. Indications that early malnutrition can cause hypochlorhydria and, in turn, lead to gastric bacterial overgrowth, gastritis, parietal cell loss, and subsequent lifelong hypochlorhydria, have yet to be followed up to identify potentially preventive early nutritional interventions. The role of particular gastric pathogens such as *Helicobacter pylori* in the causation of tropical hypochlorhydria and ultimately, in the susceptibility to cholera, is currently under study.[34]

Given the prevalence of hypochlorhydria in developing countries, and the finding of *V cholerae* serovar 01 free swimming, adherent to or inside copepods and algae,[3 4] amoebae,[5] and nematodes and their eggs,[7] it is easy to imagine how most of the human population in endemic areas acquires vibriocidal antibody titres early in life.

Pathophysiology

Study of pathogenesis of disease after colonisation has chiefly focused on the role of increased adenyl cyclase and cyclic AMP (cAMP) concentrations after cell intoxication by classic cholera toxin.[35 36] Recent elucidation of other toxins that the pathogen can produce may explain some aspects of early pathogenesis,[37 38] but seems unlikely to supplant the central importance of classic cholera toxin. Nevertheless, conceptualisation of pathophysiology should move beyond the findings of increased adenyl cyclase and cAMP. The mechanism by which cAMP might cause (and not merely parallel) the changed electrolyte and water movement in cholera patients remains unknown. In the light of evidence of multiple vibrio toxins[37 38] apparently non-A subunit[39 40] or non-cAMP related[41 42] toxic effects, and little studied differences in effects of cholera toxin on the numerous different intestinal epithelial and neuronal gut cell types, current concepts of causation seem simplistic.

In fact, evidence has mounted suggesting that adenyl cyclase and cAMP may participate only partially, transiently or indirectly in that their inhibition or antagonism have not consistently paralleled G_S or electrolyte flux changes[41 43 44] or diminished diarrhoea in experimental situations or in cholera patients.[45–47] Adenyl cyclase activity in some cholera patients may not parallel diarrhoea,[48] and in cell models adenyl cyclase activity is stimulated at low, but not at high concentrations of cholera toxin or with prolonged exposure to it.[44]

Experimental anti-secretory effects of phenothiazines were not associated with changes in ADP ribosylation, though a parallel was found with adenyl cyclase inhibition, and phenothiazine effects on calcium mediated pathways, which can also change sodium fluxes, are possibly responsible for anti-secretory effects in experimental models.[45] Studies showing modest cholera diarrhoea reduction by the cyclase antagonist chlorpromazine without tetracycline,[49] but not with tetracycline,[50] probably represent only the antibacterial efficacy of chlorpromazine, not an anti-cyclase effect on diarrhoea rate. Other cyclase inhibitors, such as chloroquine[46] are ineffective in patients.[47]

In vivo, even chloride transport inhibitors may not inhibit choleraic fluid secretion[51]; in a cell model, maximum promotion of chloride secretion did not require an increase in cAMP activity,[52 53] and the reduction of secretion can occur without diminishing cAMP activity.[54] Perhaps excised, avascular, and enervated short circuited epithelial strips or isolated cells[35 36 53] do not faithfully reflect events in intact living patients. Causal links between cAMP and the augmented villous tip hypertonicity after cholera toxin,[55] or the non-absorbability of tritiated water after cholera toxin[56] are needed if cAMP's central role is to be retained.

A cascade of cholera toxin-associated changes in G proteins, 5-hydroxytryptamine receptors,[57] possible release of prostaglandins,[58] vasoactive intestinal polypeptide[59 60] (by neuronal mechanisms) and other hormones has been shown, but not integrated. A clear chain of cause and effect explaining exactly how sodium, potassium, chloride, and water movement (and performance of related transporters) are changed to produce diarrhoea in the patient has not been shown. Whether some of the change in adenyl cyclase or cAMP activities in vivo might be a cell reaction to other effects of cholera toxin (compared with a direct cholera toxin effect) has not been ruled out. cAMP has appeared in the role of chloride secretion inducer,[35 36] absorption promoter of sodium-glucose cotransport and other substances,[61–63] protein kinase phosphorylator[64] or bystander.[44–45 52] The comparative importance in choleragenesis of other secondary messengers, or of effects of the cholera toxin cascade on cell nuclei and DNA,[65] is not yet established.

Additional cAMP pathway inhibitors with reported anti-cholera activity exist (for example, progesterone,[66] retinoic acid,[67] barium salts,[53] glucagon,[68] and serotonin antagonists),[69] which have not been clinically tested, but one must conclude that no inhibitors of adenyl cyclase, or cAMP antagonists tested have proved of therapeutic value against cholera diarrhoea. It is time to look further.

Treatment

Experimental anti-cholera agents, like acids, unsuitable for treatment but capable of removing cholera toxin affected intestinal mucosal cells, have been the only agents shown to abruptly stop the diarrhoeagenic effect of cholera toxin[70] when given after diarrhoea starts (in the dog model). This therapeutic approach has not been pursued despite the availability of potentially usable, tolerable agents such as epidermal growth factor or glutamine,[71 72] capable of speeding up intestinal cell turnover and thereby replacing toxin affected cells sooner. Concurrent antibiotics could, in such a regimen, prevent newly generated cells from being exposed to the toxin by eliminating vibrios concurrently. Neuropeptide Y seems capable of reducing diarrhoea in the cat model[60] and may merit further study, along with anti-secretory hormones found in milk (and bile) of rats and sows.[73 74] Zinc replenishment has also shown therapeutic promise,[75 76] but may interfere with oral glucose and amino acid absorption.[77]

The studies first showing that oral glucose electrolytes solution treatment (ORT) could reduce by 80% therapeutic intravenous fluid needs of cholera patients already in shock,[78] and could be used for the entire rehydration and maintenance phases in most patients not in shock[79 80] were followed by similar studies showing the utility of the amino acid glycine as a substrate.[81] Greater absorptive efficiency and reduced diarrhoea duration accompanied use of solutions containing both glucose and glycine in cholera and cholera like toxigenic diarrhoeas.[82–88] Other amino acids, for example, alanine and glutamine, can also do this[89 90] but are costlier and less available than glycine.

The practical importance of the glucose plus glycine solution in terms of reduced duration of cholera treatment and stay in hospital[81–83] has been underestimated, because of the apparent lack of reproducible advantage in non-enterotoxigenic diarrhoeas,[91] which are generally much milder than cholera and, in contrast with acute cholera,

can be treated with a very wide variety of oral fluids. Some of these studies have changed sodium concentrations and substituted polysaccharides for glucose or glycylglycine for glycine, clouding comparison with the original studies.[81-87 92 93] Some oligosaccharides may inhibit glucose absorption,[94] and polysaccharides may promote hypernatraemia.[95] Advantages seen in some studies of solutions with glucose plus glycine, or with rice powder, which is rich in glycine and other amino acids as well as starch,[96] may relate to enhancement by cholera toxin of cell sugar and amino acid absorption, perhaps mediated by cAMP.[62 63]

Subsequent studies have shown the utility, though not consistent superiority, of cereal and other complex substrates.[97] It remains unclear if the variability in amino acid content of rice used for different studies may account for variability in results of different studies of rice ORT.[98] The numerous studies of different complex substrates, ranging from rice preparations to mung bean and chicken soup[99-101] have yielded many complex solutions, which can support absorption during cholera or other related diarrhoeas, depending on local availability, but most of these studies have not been conducted in patients admitted in shock, in whom the choice of the most highly absorbable oral solutions (after initial intravenous rehydration) is of critical importance. Contradictory results have been obtained chiefly in numerous small studies unstratified by pretreatment diarrhoea rate, unblinded, and lacking sufficient statistical power to provide trustworthy conclusions. These have left uncertain the evidence that any of these solutions is truly superior to glucose ORT or to glucose and glycine ORT in cholera.[97 98] Rice malabsorption has been reported as a potential problem.[102]

In cholera in particular, the question whether the WHO ORT has optimal sodium and potassium content has been repeatedly asked, but in the interests of preserving a solution formulation usable for milder diarrhoeas as well, the WHO formula remains slighly suboptimal for cholera in its sodium concentration and generally too low in potassium concentration.[103 104] This has a potential influence on amino acid absorption[105] and hence on outcome of oral treatment studies. Studies evaluating the comparative longterm effects of more complete potassium replacement are much needed, especially for use in paediatric cholera and related non-cholera watery diarrhoeas.

Many formulas in use lack the optimal substrate to sodium ratio,[106] necessary to promote maximal sodium and water absorption. Claims (still unbacked by scientific data) that starch can promote more net absorption without osmotic penalty[107] have overlooked the issue of the substrate to sodium ratio and the fact that luminal starch hydrolysis is a prerequisite for absorption[108] and that starch malabsorption may occur.[102 109] Findings of increased water absorption from hypotonic oral solutions,[110] usable for treating low volume, short duration, non-life threatening diarrhoeas, need to include measurements of net sodium balance, which is generally noticeably negative when such solutions are used to treat cholera or cholera like diarrhoeas.[111] Similarly, base free oral solutions[112 113] may suffice in mild non-cholera diarrhoeas, but can lead to life threatening haemodynamic events during rehydration of acidotic cholera patients in shock on arrival.[114]

In recent years, many new substances have been discovered to be capable of enhancing intestinal absorptive capacity of glucose, amino acids, water or salts, and studies are needed to find out if such substances (for example, vitamin B-6, ethylacetate, 17α methyltestosterone, glucagon, epidermal growth factor), might enhance the efficacy or efficiency of ORT.[115-119] ORT remains grossly underused in many countries and improvement will require continuation of the excellent programme implementation of World Health Organisation, Unicef, USAID and other voluntary agencies, including a focus on Western developed countries.[120]

Prevention

The immune response to cholera infection, and the mechanism by which immunosuppressive effects of cholera toxin and related enterotoxins may block or diminish it in vivo[121-126] need further definition. Critical questions are the optimal modes of antigen delivery, comparative influence on protective efficacy of different routes of administration, immunogenicity and tolerability of megadoses of purer, more potent immunogens, including protein conjugates, and newer modes of adjuvantation. A mode of immunising those with group O blood type needs to be developed.[127] Despite a great deal of research,[10-13 128-130] the 80%, two year protection afforded by parenteral somatic antigen in peanut oil adjuvant still matches the best protection ever achieved by any cholera vaccine,[131] and may point to the best future approach, using more powerful, better tolerated novel adjuvants, and purer preparations containing more precisely defined key antigenic epitopes.[129] Pili,[132] outer membrane proteins,[133] and protein conjugates using enterotoxin moieties[134] are of current research interest. Live attenuated vaccines offer promise,[128] but have not yet been proved superior to older vaccines, and will depend on cold chain maintenance.

Other severe toxigenic diarrhoeas

Toxins closely related to cholera toxin are produced by non-01 vibrios, other vibrio species, *Escherichia coli*, and some strains of salmonella.[135-141] These organisms can produce a clinical syndrome of severe enterotoxigenic diarrhoea strongly resembling cholera. *E coli* causing severe cholera like disease often produce both heat labile and heat stable (ST_a or ST_b) enterotoxins.[142] Other organisms (aeromonas, etc) have been reported to produce cross reacting enterotoxins, [143] but are rarely if ever associated with diarrhoea as severe as cholera.

Details of the epidemiology and bioecology of these organisms are beyond the scope of this paper, but in general the vibrios tend to follow the pattern of a marine, estuarine or freshwater extra human niche[144 145] (for which *V parahaemolyticus* is the original paradigm). These organisms may also produce other virulence factors, including various other heat labile and heat stable toxins, shiga or shiga like toxins, haemolysins, etc. Association of outbreaks with faecally or marine contaminated food or water is common. Of special interest is the discovery of guanylin, an intestinal hormone resembling ST_a and the EAST-1 *E coli* entertoxin which is possibly the harbinger of other toxin hormone analogues to come.[146]

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