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Cholera Enterotoxin

Quo Vadis: Thirty Years After the Discoveries of Cholera Enterotoxin in India

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Preface

It is now just over 30 years since the first convincing experimental evidence was presented, by S. N. De[1] and N. K. Dutta[2], for the existence of a cholera enterotoxin (also known as choleragen or CT), and 20 years since it was first purified to homogeneity[3]. With the recognition that cholera was a toxin-mediated disease, hopes were raised that – as with diphtheria and tetanus – a toxoid vaccine would prevent the disease. Such hopes, although early supported by numerous experimental animal studies, received little encouragement from the later results of studies in adult American volunteers and controlled vaccine trials in the field using parenterally administered products (reviewed in 4–6). More recently, interest has shifted to the possible use of orally administered preparations to more closely approach the effective and lasting immunity generated during the disease itself. These include living attenuated mutant vibrio strains and concoctions consisting of large amounts of killed cholera vibrios with, or without, CT-derived antigen administered perorally. Each has been found wanting either because of excessive reactivity[7], or expense, inconvenience, or insufficient efficacy[8]. *Quo vadis*?

Introduction

The Discoveries of Cholera Toxin

In 1959, two separate groups of investigators in India practically simultaneously reported the production of relevant symptoms, *viz.*, diarrhea or outpouring of fluid into the lumen of the gut, in experimental animal models following the enteral administration of sterile cell-free products of cholera vibrios. S. N. De, a pathologist in Calcutta, in a Note that appeared in the May 30 issue of *Nature*[1], demonstrated that sterile filtrates of two Ogawa and two Inaba serotype *V. cholerae* strains grown in 5% peptone, 0.5% NaCl broth, pH 7.6, caused outpouring of fluid and distension of surgically isolated ileal loops in adult rabbits. Filtrates of the same strains grown in Dunham's (1%) peptone water medium were inactive. De used the word "enterotoxicity" to describe the activity. He also mentioned histopathological changes, including denudation of distended loops, which are now known to be artifacts produced by ischemia in the closed loop system. False positive reactions occasionally encountered in control loops in the rabbit ileal loop model were attributed by De[9] to the use of loops shorter than four inches, or to too rapid injection of samples. At the beginning of his work with live *V. cholerae*[10], enteropathogenic *Escherichia coli*[11], and *V. cholerae* culture filtrates, De was unaware that the experimental model had been introduced earlier, most notably by Violle and Crendiropoulo in 1915[12,13]. Subsequently, De and his colleagues[14,15] produced evidence dissociating the enterotoxicity from other factors (e.g., mucinase, lecithinase-C, and receptor-destroying enzyme) which had been described earlier, and also indicated that "somatic filtrate" of bacterial bodies had little or no activity.

In the meanwhile, N. K. Dutta, a pharmacologist at the Haffkine Institute in Bombay, and his colleagues reported, in a Note submitted in February 1959 which appeared in the October issue of the *Journal of Bacteriology*[2], that they could produce fatal choleraic diarrhea in infant rabbits fed multiple doses of sterile lysates of dense suspensions of *V. cholerae* strain 569B Inaba after gastric lavage. This strain had been used by Dutta earlier[16] to cause choleraic diarrhea in infant rabbits inoculated intra-

intestinally with live vibrios. In the earlier study, the virulence of the strain was found to have been enhanced following its reisolation from the heart blood of a dead infected infant rabbit. The derived strain caused fatal diarrhea in 100% of infant rabbits inoculated with 10^3 viable organisms. (In all probability, Dutta, by this procedure, succeeded in isolating a smooth virulent variant from a population of vibrios which had been degraded to become predominantly serologically rough and avirulent following repeated serial passages in artificial culture media in the laboratory over a long period of time. In the author's experience, cholera vibrios freshly isolated from patients are invariably virulent in Dutta's model and retain this property when preserved by lyophilization.) It turns out that 569B is an exceptionally good strain for producing the cholera enterotoxin (CT) [it is now universally used for that purpose by laboratories that produce substantial quantities of CT, like the PW-8 strain of *Corynebacterium diphtheriae* is for production of diphtheria toxin], but the method used by Dutta to extract it is exceptionally poor. Additionally, because the preparations used by Dutta were rich in endotoxic lipopolysaccharide, he tended to equate the enterotoxic activity with the endotoxin.

The two groups, De's and Dutta's, were intensely competitive for a time – each maintaining that theirs was the only truly relevant model for cholera. In fact, over ten years later the validity of Dutta's model was challenged by De's group[17] which claimed that the large amount of fluid that soils the ventral surface of choleraic infant rabbits was urinary rather than intestinal in origin – a notion that was rendered untenable by a subsequent study[18].

The Recognition of Cholera Toxin

Having worked with cholera vibrios since 1952 and having seen near-moribund cholera patients "come to life" within minutes of receiving appropriate intravenous fluids (in U.S. Navy Captain Robert A. Phillips' cholera ward in San Lazaro Hospital, Manila, Philippines), in 1961 I came to the realization that "there had to be a cholera toxin! It should be secreted by the vibrios! And, it must act locally! The 1959 observations of De and Dutta may indeed be relevant!"

While working with S. Mukerjee at the Indian Institute for Biochemistry and Experimental Medicine in Calcutta in 1962, I visited Dutta's laboratory at the Haffkine Institute in Bombay and observed his infant rabbit technique. Dutta, subsequently that year, visited the Walter Reed Army Institute of Research (WRAIR) in Washington, D. C. to work with Dr. Samuel Formal (then Chief of my Department at WRAIR) primarily to evaluate Dutta's lysates, unsuccessfully, in De's ileal loop model. Before then, I had started to use infant rabbits and, together with pathologists H. T. Norris and H. Sprinz, Dutta, Formal and I showed conclusively that while live *Vibrio cholerae* and Dutta's lysates produced experimental cholera, purified *V. cholerae* endotoxin (LPS) did not[19]. Formal and Dutta, however, were at the time, also unsuccessful in reproducing De's findings that *V. cholerae* culture filtrates would produce positive rabbit ileal loops[20]. Following Dutta's departure, I continued to use Dutta's model but with the presumption that Dutta was looking in the wrong place for the active principle – it should preferentially be secreted by the vibrios and the activity should be greater in the culture supernatants than in the carcasses of the bacteria. The conditions which were combined in the first experiment to test the hypothesis resulted in its success. Sterile filtrates of shaken brain-heart infusion broth cultures of *V. cholerae* strain 569B (Dutta's rabbit-passaged strain) produced fatal choleraic diarrhea when administered perorally to infant rabbits. Not only that, but the filtrates were active in a single dose (instead of the multiple doses used by Dutta), and they were still active after dilution[21]! We called the active principle "choleragen" and reported, in the same paper, that it could also be produced in chemically defined (i.e., synthetic) medium – the subject of the author's Ph.D. dissertation in 1955[22] – if the medium was supplemented with casamino acids, i.e., "syncase" medium which has subsequently been regarded as optimal for cholera toxin production. It was also observed in the same paper (which was submitted in October, 1963) that the cholera enterotoxin (CT or choleragen) was immunogenic[21].

CT was first purified to homogeneity, using exclusively Dutta's model as a bioassay[23], as reported in 1969 (10 years after the first Indian discoveries of it!). Simultaneously another protein, which appeared to be immunologically identical to choleragen but was inactive in Dutta's model, was isolated and given the name "choleragenoid"[23]. It was subsequently[24] found to be the B-subunit pentamer and the G_{M1}-binding region[20] of this bipartite enterotoxin – the prototype of a still-growing family of cholera toxin-related enterotoxins which were subsequently discovered primarily using De's model. (Most investigators still prefer rabbit ileal loops to infant rabbits.)

Thus, although, as Garfield[25] concluded, De's experimental model and De's perceptions were ultimately to have a broader and more lasting impact

(than Dutta's contribution), the fact is that cholera toxin was first purified to homogeneity and characterized without them.

The Cholera Toxins and the Cholera Toxin-Related Enterotoxins

In 1974, it was first recognized[26] that the CT ("CT-2") produced by an *El Tor* biotype Ogawa serotype cholera vibrio differed immunologically from the CT ("CT-1") which had been isolated five years earlier from classical biotype Inaba serotype *Vibrio cholerae* strain 569B[3]. Subsequent quantitative cross-neutralization studies suggested that these immuno-logical differences could be meaningful[27]. Nevertheless, all vaccine trials of the prophylactic efficacy of cholera toxoids have employed CT-1 antigen against the prevailing epidemic epitype of CT – usually CT-2[28].

Recognition that cholera was a toxin-mediated diarrheal disease also stimulated inquiry into the possibility that other enteric bacteria – including some not previously implicated as being enteric pathogens – might similarly cause diarrheal disease by means of enterotoxins. As a result, increasing numbers of CT-related heat-labile enterotoxins (LTs) have been described from strains of *Escherichia coli* of human (H) and porcine (P) origin, as well as from other enteric bacteria (see 5). [Other growing families of enterotoxins which are unrelated to the CT-related enterotoxin family; the so-called heat-stable enterotoxins, or "STs," and the cytotoxins, Shiga toxin and the Shiga toxin-like toxins (also called Shiga-like toxins, Verocytotoxins and Verotoxins), which are associated with dysentery and hemorrhagic enterocolitis, will not be considered here.] While emphasis has been placed on the immunologic relatedness of the CT-related diarrheagenic enterotoxins, their immunologic specificity may be equally as important in that enterotoxin-type specific antibodies in some instances account for a major portion of the neutralizing activity of polyclonal hyperimmune sera[27].

The LTs which have been described are structurally, functionally, and immunologically related to the CT prototype. These enterotoxins are bipartite. Like CT, these "ADP-ribosylating toxins" consist of a mature A-subunit of approximately 27,000 M_r which, when proteolytically nicked and reduced, yields an enzymatically active A_1 peptide which can hydrolyze NAD and transfer its ADP-ribose to a GTP-binding protein that regulates host (or target) cell adenylate cyclase thus directing the cell to produce excessive amounts of cAMP which results in hypersecretion of Cl^- and HCO_3^- followed by water comprising the diarrheal stool. These toxins also have a B region, which in CT was originally called choleragenoid[3], composed of five identical non-covalently associated B-subunits––103 amino acids; M_r ca 11,600 — which is also non-covalently associated with the A-subunit in the intact holotoxin. The B region, which is responsible for binding the holotoxin to host cell membranes containing the glyco-lipid G_{M1}-ganglioside and facilitating the entry of the A-subunit, is immunologically dominant and is primarily responsible for the immunological differences which have been observed among these toxins.

The amino acid (a.a.) sequences of the B-subunits of CT-1 and CT-2, of H-LT-1 [LT derived from *E. coli* strain H74-114 of human (H) origin[29]], and of P-LT [from an *E. coli* strain of porcine (P) origin[30]], which were derived from sequence analysis of the protein or predicted from the DNA sequence of the cloned structural genes (see 31), are summarized in Figure 1. Whereas the B-subunits of CT-1 and CT-2 [which were earlier shown to be immunologically distinguishable[26,27]] differ from each other by 5 amino acid residues[31a], the B-subunit of H-LT-2, originally derived from strain H10407[32], differs from that of H-LT-1 only by having Arg(13) (like P-LT) in lieu of CT/H-LT-1 His(13). Nevertheless, H-LT-2 can be differentiated from H-LT-1 by monoclonal antibodies (mAbs)[31]. So far, only one form of P-LT has been identified. Also shown in Figure 1 are the a.a. sequences of some genetically-engineered chimeric B-subunit proteins in which single (or two) a.a. residues from H-LT-B-1 are substituted for the corresponding residue(s) in P-LT-B (see 31). Additionally, the a.a. sequences of synthetic peptides ("CTPs") representing sequential portions of CT-B-1, provided with their rabbit antisera by Chaim Jacob (see 31), are included in Figure 1.

Observations on Epitopes in the Cholera Toxin-Related Enterotoxin Family

Inasmuch as successful antitoxic immunity to CT and the CT-related enterotoxins has yet to be achieved, we felt that further study of the immunology of these proteins was warranted. In the work presently described, the reactivities of a variety of antisera in our library – including hyperimmune polyclonal sera from several animal species, mAbs, anti-peptide sera, and sera from American volunteer cholera vaccinees and convalescents (generously provided by M. M. Levine and J. B. Kaper of the Center for Vaccine Development at the University of

AMINO ACID SEQUENCES OF B-SUBUNITS OF TWO CHOLERA TOXINS (CT), AND PORCINE (P) AND HUMAN (H) LTs

CT-1	Thr	Pro	Gln	Asn	Ile[5]	Thr	Asp	Leu	Cys	Ala[10]	Glu	Tyr	His	Asn	Thr[15]	Gln	Ile	His	Thr	Leu[20]
CT-2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Tyr	—	—
H-LT-1	Ala	—	—	Ser	—	—	Glu	—	—	Ser	—	—	—	—	—	—	—	—	—	Ile
P-LT	—	—	—	Thr	—	—	—	—	—	—	—	—	Arg	—	—	—	—	—	—	—

CT-1	Asn	(Asn)	Lys	Ile	Phe[25]	Ser	Tyr	Thr	Glu	Ser[30]	Leu	Ala	Gly	Lys	Arg[35]	Glu	Met	Ala	Ile	Ile[40]
CT-2	—	Asp	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H-LT-1	—	—	—	—	Leu	—	—	—	—	—	Met	—	—	—	—	—	—	Val	—	—
P-LT	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

CT-1	Thr	Phe	Lys	Asn	Gly[45]	Ala	Thr	Phe	Gln	Val[50]	Glu	Val	Pro	Gly	Ser[55]	Gln	His	Ile	Asp	Ser[60]
CT-2	—	—	—	—	—	—	Ile	—	—	—	—	—	—	Ser	—	—	—	—	—	—
H-LT-1	—	—	—	Ser	—	—	Thr	—	—	—	—	—	—	Gly	—	—	—	—	—	—
P-LT	—	—	—	—	—	Glu	—	—	—	—	—	—	—	—	—	—	—	—	—	—

CT-1	Gln	Lys	Lys	Ala	Ile[65]	Glu	Arg	Met	Lys	(Asn)	Thr	Leu	Arg	Ile	Ala[75]	Tyr	Leu	Thr	Glu	Ala[80]
CT-2	—	—	—	—	—	—	—	—	—	Asp[70]	—	—	—	—	—	—	—	—	—	—
H-LT-1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Thr	—	—	—	—	Thr
P-LT	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

CT-1	Lys	Val	Glu	Lys	Leu[85]	Cys	Val	Trp	Asn	Asn[90]	Lys	Thr	Pro	His	Ala[95]	Ile	Ala	Ala	Ile	Ser[100]
CT-2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
H-LT-1	—	Ile	Asp	—	—	—	—	—	—	—	—	—	—	Asn	Ser	—	—	—	—	—
P-LT	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

CT-1	Met	Ala	Asn
CT-2	—	—	—
H-LT-1	—	Glu	—
P-LT	—	Lys	—

CHIMERIC PLT-B
pDL-2 Glu[102]
pDL-3 Ala[46]/Glu[102]
pDL-5 Ala[46]
pDL-7 Ser[4]/His[13]

SYNTHETIC PEPTIDES

CT-P_1 8-20	CT-P_4 69-85
CT-P_2 30-42	CT-P_5 75-85
CT-P_3 50-64	CT-P_6 83-97
CT-P_7 45-64	

The validity of Asn[22] and Asn[70], originally defined by amino acid sequencing, has been questioned (Eur. J. Biochem.; 146:503, 1985).

Figure 1. Amino acid sequences of B-subunits of CT-1, CT-2, H-LT-1, P-LT, chimeras and synthetic peptides of CT-B-1.

Maryland, Baltimore) – with the antigens summarized above, were examined by a newly developed technique, "checkerboard immunoblotting (CBIB)," as described recently[33–36]. CBIB allows the simultaneous analysis of as many as 2000 antigen/antibody combinations, conveniently and reproducibly. The antigens were used in their native state, or as denatured (8 M urea), CNBr-digested, heated and/or reduced (2-ME) forms.

We also describe research in progress examining sequence-related epitopes using synthetic sequential hexapeptides spanning the 103 a.a. sequence of the CT-B-1 subunit. For this purpose, the synthetic hexapeptides were synthesized on derivatized plastic pins which conform to the configuration of a standard 96-well microtiter plate using reagents and a kit (Epitope Mapping Kit) purchased from Cambridge Research Biochemicals, Inc., Valley Stream, N.Y.[36]. The synthetic hexapeptides were tested sequentially with a variety of antibodies and the reactions were developed appropriately using secondary antibody detection systems as in enzyme-linked immunoadsorption assays (ELISA).

A representative sample of CBIB is presented in Figure 2 for illustrative purposes. In this instance, the reactions of sera from two human convalescents, who had recovered from induced *El Tor* cholera, with CT-B-1 and CT-B-2 are shown before and after adsorption of the sera with native or heat-denatured CT-B-1 or CT-B-2. Although in these cases both sera (prechallenge control sera had no activity) reacted, at the dilution used, nearly equally well with both CT-B-1 and CT-B-2, adsorption with native CT-B-1 left strong residual antibody reactive with CT-B-2 while adsorption with CT-B-2 completely removed reactivity with both B-subunit proteins. These results indicate that there was a significantly broader antibody response to CT-B-2 than to CT-B-1 in these patients. Adsorption with heat-denatured proteins did not significantly affect the reactions of the sera with the native proteins as a considerable portion of that reactivity is directed against conformational (i.e.,

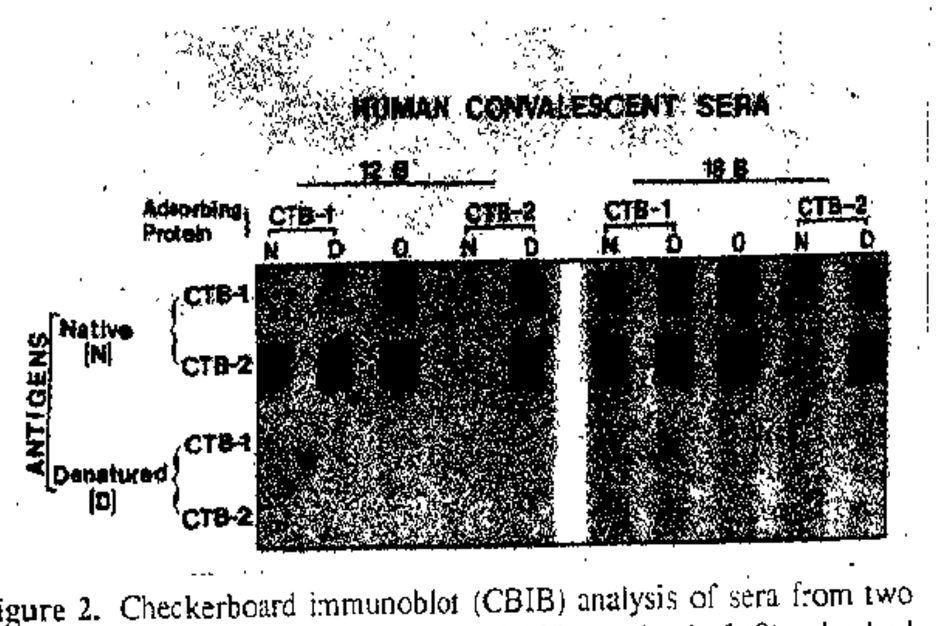

Figure 2. Checkerboard immunoblot (CBIB) analysis of sera from two volunteer cholera convalescents, 12B and 18B, unadsorbed (0), adsorbed with native protein (N) and adsorbed with denatured protein(D), tested with native (N) and denatured (D) CT-B-1 and CT-B-2.

discontinuous or non-sequential) epitopes[35]. This point is also illustrated by the lack of reactivity of the sera with the urea-denatured or CNBr-fragmented subunit proteins (Figure 2).

Results obtained with mAbs in the current work supported and extended those obtained previously in ELISA[31]. The mAbs, like their parental polyclonal sera, reacted poorly, if at all, with denatured antigens indicating that they were directed primarily against conformational (discontinuous) epitopes. Some were highly specific and recognized only the homologous antigen as was the case, for example, with a mAb against pDL-7 which (see Figure 1) is P-LT-B with H-LT-B-1 Ser(4) His(13). Apparently, in this instance, one or both of these residues together with the P-LT-B backbone created a unique epitope. Some mAbs raised against CT-1 and CT-2 likewise recognized only the homologous protein; some recognized both CT-Bs and no others, and some recognized both CT-Bs, H-LT-B-1, and pDL-7 – which all share His(13) – but did not react with H-LT-B-2 which has Arg(13). His(13) must, therefore, be involved in the formation of this epitope which differentiates H-LT-B-1 from H-LT-B-2. As the reactivity of these mAbs with the antigens is blocked by treatment of the antigens with the G_{M1}-ganglioside receptor, His(13) must be in close proximity to a G_{M1}-binding site or the epitope is masked by receptor binding.

Reactions of many different mAbs suggested the importance of a.a. residue 46. Some reactions were limited to antigens which had Ala(46) instead of Glu(46), whereas others reacted with proteins with Glu(46) and not Ala(46). In several cases like this, alteration of a single a.a. residue resulted in the disappearance of reactivity with some antibodies and the appearance of reactivity with others. We call these epitopes which appear and disappear with changing a.a. residues "complementary epitopes". Several examples were encountered[35] and, in some instances, reactivity was blocked by treatment of the antigen with G_{M1}-ganglioside prior to adding the mAb, while in other instances it was not. The results suggested that several different regions of the B-subunit structure contribute cooperatively to G_{M1}-binding. In some instances, studies with the genetically engineered chimeras revealed that having the proper residue 46 was essential, but not necessarily sufficient, for expression of an epitope: cooperation of other distal residues was required. Additional mAbs reacted promiscuously indicating the presence of conserved epitopes in all of the antigens regardless of their a.a. differences.

In contrast to the mAbs examined, polyclonal antisera frequently reacted with denatured forms of the antigen. Further, despite the immunological relatedness of all the proteins studied, even hyperimmune sera reacted with some degree of specificity. For example, an equine anti-CT-B-1[37] reacted promiscuously, but much more strongly with the CT-Bs and with chimeras with Ala(46). Conversely, a goat anti-P-LT reacted very weakly with the CT-Bs and H-LT-B.

Polyclonal rabbit antiserum raised against synthetic peptide CTP-1 (residues 8–20 of CT-B-1, Figure 1) was very specific for denatured CT-B-1: it did not react with CT-B-2. As the only difference between CT-B-1 and CT-B-2, in that region, is His(18) vs Tyr(18) (Figure 1), the definitive residue must be His(18) and, as the antibody does not recognize the native antigen, this epitope is not exposed in the B-subunit pentamer. Anti-CTP-3 and anti-CTP-7, raised against conserved regions of the B-subunit proteins (Figure 1), reacted vigorously with all the denatured antigens tested, but little reactivity was detected with the native proteins. Thus, although as demonstrated in previous neutralization studies[38], this sequence contains, or is part of, a surface epitope, the antigen is apparently more exposed when the protein is unfolded.

The serological responses of American volunteers who had recovered from induced cholera due to *El Tor* biotype *V. cholerae* varied among individuals. Some had strong promiscuous responses, but generally the reactions were noticeably more vigorous with CT-B-2 than with CT-B-1 (as in Figure 2). Several sera were more reactive with the CT-Bs and pDLs 2, 3 and 7, and markedly less reactive with the LTs and pDL5. Reactions of human convalescent sera were largely directed against conformational determinants and were reduced by G_{M1}-treatment of the

antigens. American volunteers who had received a genetically-engineered *ctx* A^-B^+ candidate mutant vaccine strain, CVD 103[39] derived from strain 569B Inaba (i.e., CT-B-1), exhibited antitoxic responses which were significantly weaker[35] than those from convalescents from induced cholera. This may be because CVD 103 colonizes poorly.

Epitope scanning studies using sequential hexapeptides revealed a large number of sequence-derived epitopes particularly when certain hyperimmune sera were used. For example, a rabbit polyclonal anti-CT-B-1 serum (Figure 3) recognized 8 distinct sequence-derived epitopes spanning the CT-B-1 protein. However, most of the sequence-derived epitopes appear to be buried determinants as adsorption of the serum with native CT-B-1 hardly affected the pattern of reactivity whereas adsorption with cyanogen bromide (CnBr)-fragmented protein reduced most of the reactions. In this regard, CnBr-CT-B-2 behaved similarly to CnBr-CT-B-1 as the immunological differences between the two toxins are primarily conformational. None of the mAbs tested reacted with the synthetic hexapeptides, as expected, since they, as well as their parental polyclonal sera, did not react with CNBr-cleavage products of the B-subunit proteins[35]. Sera from the human cholera convalescents tested were also more reactive with conformational epitopes and did not give distinctive patterns of reactivity with the synthetic hexapeptides.

Discussion

The 30 years since the discoveries of CT have been disappointing in the sense that they have not yet led to the development and deployment of a practical, non-reactogenic, economical and effective vaccine against cholera. However, CT has provided other, earlier unexpected benefits. It is the prototype of an ever-increasing number of CT-related, and CT-unrelated, enterotoxins which, on a global scale, are probably vastly more important than CT is *per se* in terms of morbidity and mortality. According to World Health Organization approximations[40, 41], more than 4 million children under the age of 5 die of diarrheal disease each year. Although results vary in different studies, perhaps as much as 50% of these

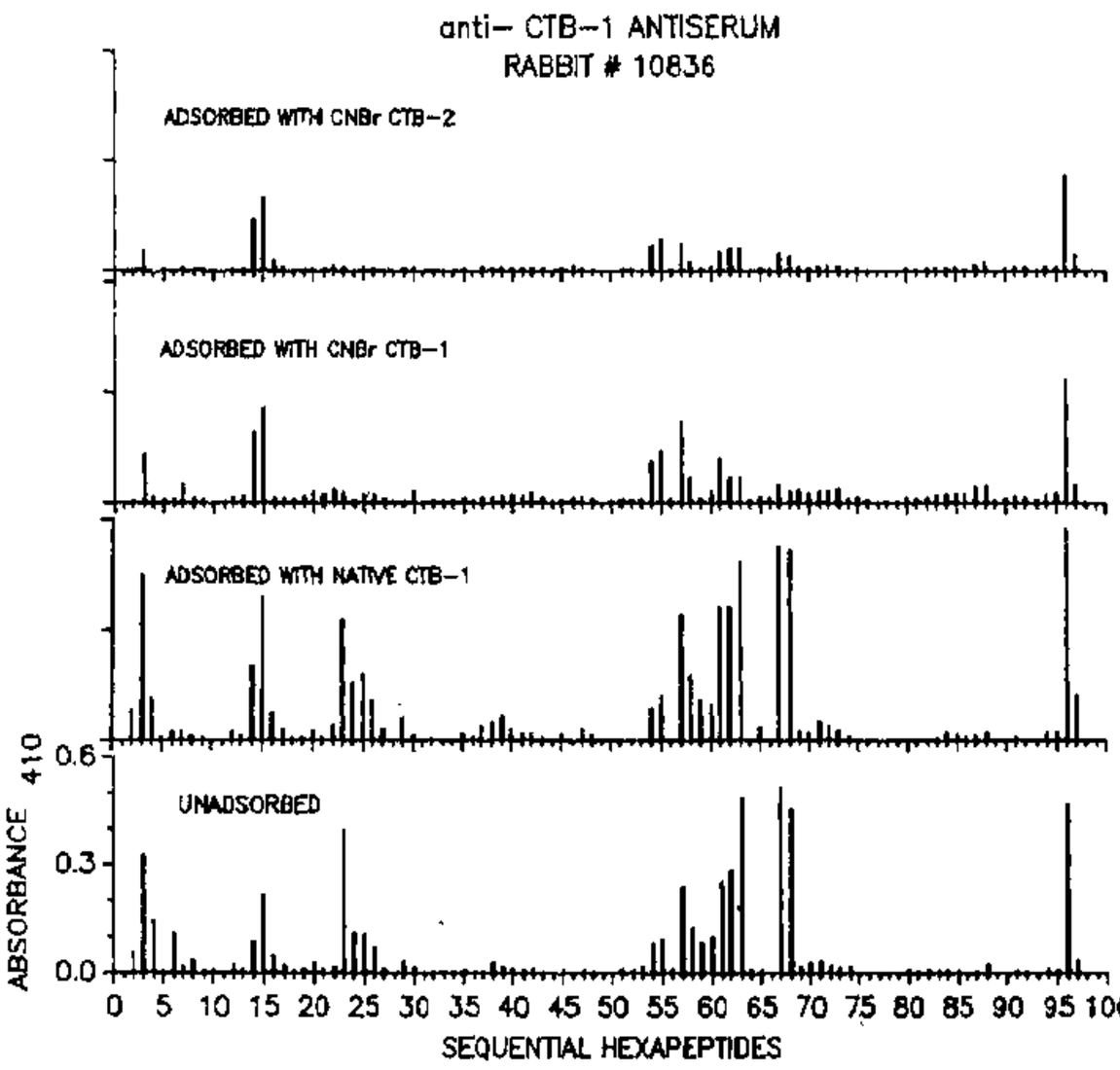

Figure 3. Epitope scanning using a hyperimmune polyclonal rabbit serum raised against CT-B-1 with sequential hexapeptides of CT-B-1. The vertical bar (Y-axis) over hexapeptide 2 (X-axis) reflects the reaction (absorbance @ 410 nm) between antibody in the serum and the hexapeptide representing a.a. residues 2–7. The higher bar over hexapeptide 3 reflects reactivity with peptide 3–8, etc. The size of the sequence epitope (trimer, tetramer, etc.) is inversely related to the number of reactive consecutive hexapeptides. The effects of pre-treatment (adsorption) of the antiserum with native homologous antigen (CT-B-1) and cyanogen bromide (CnBr)-fragmented CT-B-1 and CT-B-2 are also shown.

are enterotoxin-related (particularly involving toxinogenic strains of *E. coli*) – the remainder being due to viruses (rotavirus in particular) and eukaryotic parasites. It is presently possible to significantly reduce the mortality rates by appropriate case management. This is based upon oral rehydration therapy (ORT) using oral rehydration salts (ORS) solutions, some of which can reduce stool output, duration of diarrhea and ORS requirements[8, 41]. This lifesaving measure is increasingly being applied in the lesser developed countries which bear the major burden of life-threatening diarrheal disease. Hopefully, studies on the modes of action of the CT-related and CT-unrelated enterotoxins will yield rational and specific methods of treatment which will actually stop the fluid and electrolyte losses. Ultimately, however, it is widely recognized that prevention is better than treatment.

That solid and long-lasting immunity against cholera is feasible has been established unequivocally by studies in American volunteers (see 4–7). Volunteers who had recovered from induced cholera were resistant to subsequent challenge with virulent *V. cholerae* for up to three years – the longest period tested. Thus the disease itself is an immunizing process; one in which the host is presented with all of the products of cholera vibrios, including the lipopolysaccharide, CT and other extracellular and cell-associated products and membrane proteins, elaborated by the vibrios growing *in vivo* on the surface of the small bowel. It is not yet certain which of these products, or combination of products, or method of presentation, or combination of product and presentation, is most important. *In vivo* grown cholera vibrios produce specific outer membrane protein antigens which are not produced under ordinary culture conditions *in vitro*[42,43], and this could be a factor. It has also been shown[44] that live vibrios are much more effectively sampled and translocated than killed vibrios by the M cells overlying Peyer's patches and this, too, could be highly significant. Interestingly, immune mechanisms which do not involve CT antigen can be protective, as shown by volunteer studies with a genetically-engineered CT-deletion mutant[7]. Whether the protection would be stronger and more durable with CT antigen is not clear. Likewise, the question may be raised of whether or not effective and lasting immunity can be attained by an appropriate, properly presented CT antigen by itself. In this regard, it is particularly interesting and pertinent to note that mutant strains of *V. cholerae*, which cannot produce complete CT, still cause diarrhea to a greater[7] or lesser[45] degree depending on the strain. From this, it may be inferred that *V. cholerae* has at least two mechanisms, in addition to CT, for causing diarrhea. One should, therefore, not expect that CT antigen, by itself, can protect against diarrhea caused by cholera vibrios although it may, indeed, protect against life-threatening diarrhea by itself; or, in concert with other antigens, it may help protect against infection, diarrhea and life-threatening diarrhea by affecting "colonizing ability"[46]. In fact, in volunteer studies (see 6), whereas massive doses of a killed whole cell vaccine administered perorally together with small doses of procholeragenoid [procholeragenoid is a high molecular weight, highly immunogenic toxoid produced by heating pure CT under controlled conditions[47, 48]] did not significantly reduce the attack rate of diarrhea in challenged volunteers, the total diarrheal stool volume (1.6 in vaccinees vs 9.4 liters in controls) and the total number of loose stools (6.5 in vaccinees vs 22.0 in controls) were significantly reduced. One may therefore conclude that antitoxic immunity did, indeed, reduce the severity, if not the incidence, of cholera diarrhea. In earlier experimental animals studies[48], it had been demonstrated, for the first time, that perorally or parenterally administered choleragenoid (B-subunit protein) or procholeragenoid induced immunity against subsequent intestinal challenge with virulent *V. cholerae*.

Another question is whether antitoxic immunity generated by CT-specific antigen would extend to diarrheal disease caused by the more numerous and significant CT-related enterotoxins. Based upon the results of the field trial of orally administered combined whole cell (WC) cholera vaccine and cholera B-subunit (BS) protein (from CT-1), Clemens *et al.*[49] concluded that there was a statistically significant reduction in the occurrence of diarrhea caused by LT-producing *E. coli* during the first three months following vaccination but none during the ensuing nine months. As the number of cases was very small and there were unexplained inversions of the results [e.g., as in the previous study[50], inclusion of B-subunit apparently increased the incidence of diarrhea in some groups[51]], the real significance of the observations is not clear. They did, however, claim that the short-term protection was particularly notable against life-threatening diarrhea. In the previous study[50], it is noteworthy that the protection observed was better against classical than El Tor cholera.

Results of the present (admittedly incomplete) work and previous studies[27, 31] suggest that type-specific epitopes may play a more significant role in effective immunity than was previously recognized. Jacob and his colleagues[38] achieved some degree of cross-neutralization with antisera prepared against conserved peptide CTP-3 (residues

50–64 of CT-B-1) but the titers of the serum were not particularly potent and they varied among the CT-related enterotoxins used. The same sera bound poorly to undenatured, and much better to denatured CT-B-1 and the related proteins in our laboratory (35 and current observations). This indicates that although the amino acid sequence is totally conserved (except for Ser(54) in CT-B-2), the epitope is largely buried in the native proteins. This is even more evident with anti-serum against CTP-1, raised against partially conserved CT-B-1 residues 8–20, which reacted poorly with the homologous protein[38]. In our hands (35 and currently), anti-CTP-1 reacted only with the denatured homologous protein and not at all with the related proteins probably because of the heterogen-eity at residue 18. However, the anti-CTPs reacted well, and appropriately, with synthetic hexapeptides[16].

In a practical sense, the ''immunologic relatedness'' of the family is probably overemphasized. To attain effective broad spectrum immunity against members of the CT-related family, it may be necessary to employ a combination of antigens or synthetic peptides which will reflect epitopes which are available on the surface of the native proteins and which will stimulate neutralizing antibody. Further studies are needed to define the nature of such epitopes. As some mAbs reacted promiscuously with native, but not denatured, B-subunit proteins, there may be conserved conformational (i.e., discontinuous) epitopes which would be good targets for further study. However, the arbitrary selection of a shared a.a. sequence for a synthetic peptide vaccine is not likely to be sufficient.

To attain effective immunity in the population largely at risk, i.e., children in lesser developed countries, the possibility of passive immunization by appropriately specific preformed bovine milk antibody administered perorally in infant feeding formula should be considered[52, 53].

Summary

Using a variety of techniques and CT-related products including: enzyme-linked immunosorption assays (ELISA); a newly introduced technique which we call ''checkerboard immunoblotting (CBIB)''; genetically-engineered chimeric B-subunit proteins in which individual amino acid residues of P-LT-B have been replaced by corresponding residues of H-LT-B; synthetic peptides representing sequential amino acid sequences of the immunodominant B-subunit protein of CT; monoclonal antibodies; polyclonal hyperimmune sera; and sera from American volunteer cholera convalescents and vaccinees, we have attempted to define immunologically significant epitopes in the CT-related enterotoxin family. The results indicate that individuals vary in their responsiveness to specific epitopes. Sera from *El Tor* cholera convalescents, for example, reacted better with CT-2 than with CT-1. Single amino acid substitutions can have major effects on epitope expression – either negatively or positively. Some immunological responses are directed against hidden (occult) a.a. sequences which may not be relevant to prophylactically effective immunity, whereas others are directed to conformational (i.e., discontinuous) epitopes expressed by native, and not denatured, B-subunit proteins. ''Epitope scanning,'' using an array of overlapping sequential synthetic peptides, offers the possibility of identifying significant specific or cross-reacting continuous epitopes. These observations may be pertinent to the development of effective antitoxic vaccines against cholera and the CT-related enterotoxic enteropathies. Passive oral immunization with preformed bovine milk antibody may be a useful measure in protecting children against diarrheal disease.

S. N. De, were he here today, might share our disappointment that the discoveries of cholera toxin 30+ years ago have not yet provided a solution to the cholera problem, but he would doubtless share our excitement about the world that has been opened for us, as well as our hopes and expectations that the solution lies ahead.

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