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COMMENTARY

ESCHERICHIA COLI ENTEROTOXIN

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Escherichia coli, the microorganism, which colonizes the intestinal system of animals and man soon after birth, was isolated first from faeces by Escherich in 1885. It contributes continuously to new knowledge in molecular biochemistry and genetics. The wall of *E. coli* as well as of other gram negative bacteria contains lipopolysaccharides (endotoxins). The chemistry, biology and clinical significance of endotoxins is the object of continuous research and several reviews have appeared in the past twenty years. The lipopolysaccharides, complexes with lipids and proteins form the outer membrane of *Escherichia coli*. Lipopolysaccharides consist of three regions—the O-specific polysaccharide, determining the many different O serotypes of *E. coli*. This part is linked to the core polysaccharide, common for the serotypes. The core is linked through a 2-keto 3-deoxyoctanate trisaccharide to the lipid component—lipid A. Lipid A is the part of the moiety which possesses the pharmacological activity of endotoxin and considerable progress has been achieved in elucidating its chemical structure [1]. The importance of endotoxin in the fatal outcome of gram negative rod bacteremia in humans (endotoxin-shock) is widely accepted [2]. In this commentary the main attention will be paid to another challenging site of the *Escherichia coli* story.

Since many years *Escherichia coli* has been accused as the cause of neonatal enteric infection in infants and domestic animals. The still current concept presumes specific so called enteropathogenic O serotypes of *Escherichia coli*, different for each species to be the cause of the majority of neonatal enteric infections [3, 4] The first challenge of this established view came from Smith and Halls [5, 6]. They published a detailed analysis of infections by various *E. coli* strains in different species of domestic animals, which caused diarrhea mainly in calves and piglets. They demonstrated that not only the bacterial cultures but also their filtrates produced fluid accumulation in ligated loops of the small intestine. The product which caused fluid accumulation in the intestinal loops was found in the cultivation media i.e. extracellular. Endotoxin from these culture did not cause fluid accumulation in the intestinal loops. Thus Smith and Halls came to the conclusion that *E. coli* strains which produce diarrhea must have the ability to proliferate in the small intestine and must produce an enterotoxin, which causes the fluid accumulation. Moreover Smith and coworkers demonstrated that the production of enterotoxin depends on a transferable extrachromosal plasmid, called Ent. [7, 8]. This discovery prompted a great deal of research both on the pathogenesis of *Escherichia coli* induced enteric infections and on the nature and properties of enterotoxic products of *Escherichia coli*. The plasmid bound production of *Escherichia coli* enterotoxin was confirmed in strains from piglets [9], and from humans [10]. A heat labile toxin of *E. coli* of chick origin lethal for chicken but without enterotoxic activity in the rabbit loop assay has also been described [11]. According to the actual stage of knowledge *E. coli* produces one heat labile and one heat stable enterotoxin [12]. The identification and current differentiation is so far done by means of biological assays.

Methods for toxin assay

1. In vivo *assay on ligated intestinal loops*. This method is derived from the biological assay of cholera toxin. *Vibrio cholerae* cultures [13] and/or filtrates (toxin) [14] introduced into ligated loops of the small intestine of rabbits produce fluid accumulation. The same approach was used to demonstrate enteropathogenicity of *E. coli* cultures isolated from humans [15, 16] and from domestic animals [17]. For the reproducibility of this type of experiment the small intestine of the same species from which the *Escherichia coli* had been isolated is preferable. The accumulation of fluid in rabbit intestinal loops with *E. coli* cultures is less reliable, though the microorganisms isolated from different species may multiply [6, 17].

On the other hand rabbit intestinal loops are very suitable for the demonstration of fluid accumulation after the administration of *E. coli* filtrates (the *E. coli* isolated from several species), with enterotoxic activity [18]. On the ligated rabbit loops the differentiation between heat stable and heat labile toxins is demonstrated by difference in time when the maximum fluid accumulation is detected. For the heat stable toxin this is 6 hr, for the heat labile 16–18 hr, after exposure.

2. *Infant mouse assay*. For the heat stable toxin assay an *in vivo* method using infantile mice has been developed [19]. The product is transmurally injected into the stomach of four infant mice (2–4 days old) and after 4 hr the whole intestine and residual bodies are removed and weighted. The intestine to remaining body ratio is determined and corresponds to fluid accumulation.

3. *Cell culture assays*. On adrenal cell monolayer tissue cultures a method for the assay of heat labile enterotoxin has been developed by Donta and coworkers [20]. The toxin induces 3-ketosteroid production and morphological changes (rounding of the cells) are evaluated.

B.P. 26/12 A

Chinese hamster ovary tissue cultures are also suitable for biological assay of the heat labile *E. coli* toxin. In this case an elongation of the cell is observed [21].

4. *Vascular permeability change assay.* For the assay of enterotoxic *E. coli* filtrates a method known to pharmacologists from assay of changed local vascular permeability was introduced [22]. The filtrates are injected to rabbits intradermally and intravenously injected Evans blue is used for the detection of vascular permeability changes (bluing). For *E. coli* filtrates from India and Bangladesh the results were in good agreement with the intestinal loop method. Results with filtrates from *E. coli* strains isolated from patients in the U.S.A. were less reliable. Findings by this type of assay are further complicated by recent studies [23]. Besides the classical "bluing" factor a pale area has been observed, which can, with partial purification of the *E. coli* filtrates be divided from the "bluing" activity and has been named by Finkelstein "blanching" factor, which is present in a heat stable fraction (see below) [24].

Nature of heat labile Escherichia coli *enterotoxin*

Unlike cholera toxin the *E. coli* enterotoxins have not yet been prepared in purified form. The first attempts [25] to purify the heat labile enterotoxin after filtration through XM 300 membranes and fractionation by various sepharose columns were not successful, as toxin activity was present in fractions collected over a wide-area of the gel. Also Jack and coworkers [26] were unable to separate from the partially purified heat labile *E. coli* enterotoxin endotoxin activity.

Recently several groups have prepared heat labile enterotoxin of *E. coli* of higher purity. Dorner *et al.* have used an *E. coli* strain of porcine origin producing thermolabile toxin. The purification proceeded in five steps. I Crude supernatant, II Bio/gelagarose 5, III Sephadex G-75, IV Preparative isotachophoresis, V Repeated isotachophoresis, with a different ratio of the molarities of the leading ion or a different leading electrolyte. The final material from the isotachophoresis column was concentrated by vacuum dialysis and subjected to gel filtration on Sephadex G-50. The molecular weight of the toxin was found to be 102,000 daltons. Micrograms were active in the rabbit and pig small intestine loop tests and less than 1 μg in the test of adenylatecyclase stimulation (broken cells of myocardial tissue) and in the rabbit skin test [27, 28]. Further attempts to purify the toxin come from the Finkelstein group [23]. They used an *E. coli* strain isolated from a patient with clinical cholera-like symptoms [29]. The cultivations were performed in two different media. With syncase medium and casamino acid (the medium for optimal production of choleratoxin) the microbe-free supernatant was further processed by $(NH_4)_2SO_4$ precipitation, gel filtration, ion exchange chromatography and disc gel electrophoresis. It was possible to separate an active region which produced in the rabbit skin assay the typical bluing effect (hence bluing factor). It is histologically characterized by mixed cellular infiltrate beginning 18 hr after intradermal injection accompanied later by edema. Resolution started at 48 hr. Changes appearing after intradermal injection of the "blanching factor" started early (already after 3 hr) and after 18 hr numerous mecoses were seen. The process intensifies during the 72 hr of observation. In contrast to effects by "bluing factor" there was little edema. The blanching factor is heat stable the bluing factor heat labile. The bluing factor was active on the Chinese hamster ovary assay. The previously described product of Dorner had the same activities, but immunologically both products were different, the molecular weight of the Finkelstein product was around 80,000 daltons and neither toxin precipitated with antiserum to the other. The yield of the toxin was, with the same fermentation technique, less than 1/100 of cholera enterotoxin.

When the cultivation technique was changed to trypticase soya broth (i.e. the same medium as used by Dorner for his strain of porcine origin) and processed in the same way as previously, blanching activity could be again separated from bluing activity. In this case antiserum reactivity with Dorners toxin could be demonstrated. Otherwise, depending of the original *E. coli* strain, and some differences in processing the products vary considerable with regard to molecular size, electrophoretic mobility and immunological reactivity. All these products though of low specific activity in comparison with that of *V. cholerae* enterotoxin are neutralizable to high titer by antibody to choleragenoid or by antibody to the B chain of cholera enterotoxin. It had been demonstrated previously, that high dilutions of equine antiserum to choleragenoid neutralized crude heat labile enterotoxin from porcine and human *E. coli* origin [9, 30]. Evans and coworkers [31] used a different technique to gain *E. coli* enterotoxin. They release enterotoxin by means of polymyxin B, from intact *Escherichia coli* cells, remove the cells by centrifugation, precipitate the toxin by ammonium sulphate and process further by gel filtration. For the assay of biological activity they use their method of the vascular permeability factor assay [22] and the adult rabbit loop assay, originally used for *Vibrio cholerae* and cholera toxin [13].

The product was positive in both tests and its molecular weight was determined to be approximately 20,000 daltons. The product was neutralized both by antienterotoxin sera, anticholeragen and anticholeragenoid sera. When the polymyxin-B release was performed with 18 instead of 6 hr *E. coli* cultures, the main activity was found with material presenting higher molecular weight, around 40,000 daltons. The low molecular fraction could not be found in supernatant fluid of either 18 or 6 hr *E. coli* cultures.

In a recent paper the same group [32] used several steps to purify the polymyxin B released product. Electrophoretic analysis, backextraction with ammonium sulphate, agarose chromatography, DEAE-Sephadex chromatography and finally affinity chromatography with Affi-Gel 202. The final product of the 20,000 dalton enterotoxin produced a single precipitin band in the presence of several different antisera against crude preparations of the toxin.

The same was produced with cholera toxin and choleragenoid antiserum. Dafni and Robbins [33] approached the problem in a different way. They concentrated the supernatant of *E. coli* cultivated on a medium containing casaminoacids by vacuum dialy-

sis. The concentrate was active in the rabbit small intestine loop assay. The concentrated filtrate was applied to an affinity column prepared with IgG antibodies to the toxin of *Vibrio cholerae*. Elution of the retained material with 3 M KCNs yielded a non-enterotoxic protein that precipitated with antiserum to *V. cholerae* toxin and had three major protein components on sodium dodecyl sulphate gels. After treatment with 2-mercaptoethanol two protein components were found. Only elution with 5 M guanidine yielded one protein with enterotoxic activity in the ileal loop and adrenal cell test. The toxin precipitated with *V. cholerae* toxin. Elution with KCNS led to loss of enterotoxic activity. The method has the advantage of a single step procedure in the preparation of *Escherichia coli* enterotoxin. But with other eluents (KCNS) the product loses its enterotoxic activity.

The purification of the *Escherichia coli* heat labile enterotoxin is evidently now in the center of interest of several groups. Its preparation in pure, crystalline form and unequivocal characterization, in spite of the recent progress, is still the matter of further research.

Mechanism of action of Escherichia coli *heat labile enterotoxin*

Following the discovery about adenylate cyclase stimulation in the mucosa of the small intestine by cholera toxin [34, 35] the same was demonstrated for heat labile enterotoxic filtrates from *Escherichia coli* of human [36] and calf [37] origin. Like cholera toxin [24] the enterotoxin stimulates adenylate cyclase also in other tissues [36–38]. This leads to active stimulation of secretion from the laminal to the luminal side of the small intestine [39]. The fluid accumulation in the gut due to *Escherichia coli* enterotoxins and the net flux preponderance from cell to lumen has been demonstrated by several groups for heat stable and heat labile *Escherichia coli* enterotoxin *in vitro* and *in vivo* [40–42]. The changes are functional. The loss of water and electrolytes including sodium bicarbonate leads to dehydration, acidosis and in severe cases to cholera like dehydration shock.

How does the *Escherichia coli* heat labile enterotoxin act on the inner surface of the cell to activate adenylate cyclase?

This is a field where challenging new knowledge is steadily accumulating. *Escherichia coli* heat labile enterotoxin belongs to the toxins, which are fixed by ganglioside to the cell membrane. The gangliosides serve as surface membrane receptors and for cholera toxin it has now been quite firmly established, that the specific ganglioside is the G_{ML} the monosialoganglioside, galactosyl-*N*-acetylgalactosaminyl-*N*-acetylneuraminyl-galactosyl-glucosyl-ceramide [43, 44]. There has been considerable discussion whether the same ganglioside is the membrane receptor for both toxins [45–47]. According to recent evidence this is really so. All communications agree that labile enterotoxin is fixed on the membrane to ganglioside G_{M1} but there are some doubts whether this binding is specific as for cholera toxin. In cultures of Y1 adrenal cells the binding site of both toxins is the same, but probably not G_{M1} ganglioside though it may be close to it [20]. For choleragen the role of G_{M1} as the membrane receptor has been demonstrated repeatedly by several groups [44, 48–50].

Moss and coworkers [51] have shown on chemically transformed mouse fibroblasts, which do not contain gangliosides, that the stimulation of adenylate cyclase is dependent on the presence of G_{M1} and with increasing cellular content of G_{M1} the rate of rise of intracellular c-AMP in response to choleragen was increased. An analogous experiment with heat labile *Escherichia coli* toxin would be helpful to decide, whether the receptor site for this toxin is the same as for choleragen. The binding of cholera toxin to ganglioside in the membrane is followed by redistribution of surface components of the membrane by lateral movement of the toxin-ganglioside complex in its fluid phase [52, 53]. Evidence for such redistribution (patching and capping) on lymphocyte has been brought by the binding of the 56,000 dalton subunit of cholera toxin which is considered to be the binding part of the toxin to ganglioside.

For cholera toxin it is supposed by Fishman *et al.* [44] that following the binding of subunit B to ganglioside, the toxin complex undergoes a conformational change, that promotes dissociation of the complex and entry of the A subunit into the plasma membrane. This part is necessary in the cell for the activation of adenylate cyclase. Indeed, as demonstrated by Moss *et al.* [51] ganglioside for fixation of cholera toxin or heat labile *Escherichia coli* enterotoxin is necessary for activation of adenylate cyclase only in intact cells, while in cell homogenates the adenylate cyclase stimulation takes place without the presence of ganglioside.

The adenylate cyclase stimulation requires NAD, ATP and a soluble protein of unknown structure [54]. This finding led to elegant experiments [55] which not only give the same results as with cholera toxin, but contribute to partial elucidation of the structure of heat labile *Escherichia coli* enterotoxin. Polymyxin released *Escherichia coli* heat labile enterotoxic material were peptides of about 20,000–40,000 daltons. Like A_1 cholera peptide *E. coli* enterotoxin caused immediate dosis dependent rise in membrane-bound adenylate cyclase activity. A mixture of both toxins had a greater activation effect. Antisera to cholera toxin completely prevented the activity of both toxins. When however the reaction already had started antisera were inactive. The lack of reversibility reflects an enzymatic mode of action for both toxins. Electrophoresis on polyacrylamide gels with 0.1% sodium dodecylsulphate revealed a one peak activity at approximately the same position as cholera peptide A_1 (23,000 daltons).

This peptide activates adenylate cyclase in the same way as the cholera A_1 peptide. The *Escherichia coli* peptide is inactivated by cholera toxin antibodies. Thus the two peptides are probably very similar if not identical. *Escherichia coli* enterotoxin is neutralized both by antisera to choleratoxin and choleragenoid (anti B) [56]. This speaks for the presence of another component of the enterotoxin molecule which has some similarity to the cholera B component. There are however differences in the binding of the two toxins to ganglioside [55]. Probably the binding to ganglioside in the membrane of the intact cell is the reason for the lag period before adenylate

cyclase activity is stimulated by *E. coli* enterotoxin. During this period apparently the toxin fragment necessary for this stimulation is dissociated and enters the cell. In general diarrhea due to *Escherichia coli* enterotoxins is not as severe as in cholera [24]. The difference in binding to the surface of cells might be an explanation for this. Research in the field will certainly in the near future bring further knowledge about the *Escherichia coli* heat labile enterotoxin.

Heat stable Escherichia coli *enterotoxin*

Heat stable enterotoxic activity was first described by Smith and Halls [5]. Such material from *Escherichia coli* of porcine origin has been found to be heat stable, dialysable and non antigenic [26, 56, 57]. According to earlier findings the heat stable toxin is dialysable and of small molecular weight (1,000–10,000 daltons) [58]. *Escherichia coli* heat stable toxin production *in vivo* has been demonstrated on gnotobiotic colostrum deprived piglets. Heat stable enterotoxin *Escherichia coli* strains were administered. The centrifugated supernatants of faeces revealed enterotoxic activity in the infant mouse assay [59]. *Escherichia coli* strains producing only heat labile, heat stable or both toxins have been isolated from humans [60]. It is now evident that the production of heat stable *E. coli* enterotoxin of porcine and human origin is also plasmid bound [61, 62]. It remains to be established whether the plasmids of *E. coli* from human and animal origin differ, a possible important factor for the development of diarrhea in the relevant species. In agreement with Kantor [12] we were unable to find adenylate cyclase stimulating activity with heat stable filtrates (unpublished observation). The nature and mechanism of action of the heat stable toxin waits still for elucidation. Although heat stable enterotoxin is found more frequently than the heat labile there is so far much less research on the molecular level.

Pharmacology of Escherichia coli *enterotoxins*

Both enterotoxins cause accumulation of water and electrolytes in the gut as has been described previously. This is not accompanied by any morphological microscopic changes. Electronmicroscopic analysis confirms that the toxin producing microbes adhere to and do not penetrate the epithelial cell, but revealed an increased formation of autophagosomes. The border of the epithelial cells of the small intestine of rabbits remained intact [63].

Metz *et al.* [64, 65] observed impaired rythmicity of isolated rabbit small intestine segments after the administration of heat labile filtrates from enterotoxic *E. coli*. The changes remind Luciani periods. Donta *et al.* [66] demonstrated steroidogenesis in adrenal cell cultures induced by the heat labile toxin, Kantor [12] has proposed for *Escherichia coli* heat labile enterotoxin an important role as a probe for systems regulating cell division and the study of adenylate cyclase kinetics. Adenylate cyclase stimulation leads to increased intracellular levels of cAMP and these to a reduction in the rate of division of mammalian cells [67]. This could explain the pregnancy interrupting potency of cholera and *Escherichia coli* enterotoxin [68, 69].

Although originally prostaglandins were supposed to play an essential role in cholera diarrhea [70], evidence is accumulating, that prostaglandins, though possessing some modulating role, are not essential for enterotoxin activity [12]. As progress is made in the preparation of more purified *E. coli* enterotoxins certainly other sites of investigations will gradually appear.

The changing concept of diarrhea induced by Escherichia coli

On the basis of the just described newly acquired knowledge about the production of plasmid bound, thus transferable production of *Escherichia coli* enterotoxin, it is necessary to challenge views which still persist in human and veterinary practice and in textbooks [70]. On the basis of experience with epidemic outbreaks of severe diarrhea in infants and neonate animals in the first weeks of life the view was accepted that diarrhea is due to infection with a comparatively small number of *Escherichia coli* O serotypes, distinct for each species. This view challenged for infants by Gordon *et al.* [72], has been since then challenged by others, also in the explanation of the so called travellers diarrhea in adults [24, 73–76].

Our own experience with serotyping of many hundred strains of *Escherichia coli* of human, calf, hen, chicken and egg yolk origin agree with these findings. Over sixty different O serotypes and many non typable strains were isolated. So called human enteropathogenic strains were found in calves, hens and chickens and vice versa [77, 78, to be published]. The expression "enteropathogenic" should in this respect be replaced by "enterotoxic" related to the production of enterotoxin. From humans and domestic animals *Escherichia coli* strains producing heat stable or heat labile or both toxins have been identified (at least in enterotoxins of human or calf origin) [60, to be published]. Sufficient enterotoxin production needs the fixation of *Escherichia coli* to the wall of the small intestine. In this the K factors play a role. They are again plasmid bound and transferable. The K88 antigen is important for pigs, the K99 for calves and recently a similar factor was found to be important for humans [79–82].

Rutter *et al.* [83] have recently brought some evidence that the K88 antigen is adhesive only in some piglets and on the basis of their experiments they came to the conclusion that adhesiveness or not adhesiveness is phenotype dependent thus genetically determined. If we add that the H group testing of *Escherichia* coli strains is in its beginning [84] the complexity of the whole problem is apparent in spite of the great progress in knowledge. It is also understandable why effective immunisation procedures are not yet definite. As there is considerable crossimmunity between *E. coli* and *V. cholerae* enterotoxins, effective antitoxic immunisation with toxoid against cholera could also to some degree protect against *E. coli* enterotoxic activities. This would be of outstanding importance for developing countries and of some value for modern husbandry.

As soon as the *E. coli* enterotoxins were discovered the possible production of enterotoxins by other microbes became a tool for research. Indeed progress in this field is in rapid development. *Clostridium per-*

fringens [85], *Pseudomonas aeruginosa* [86] and *Shigella dysenteriae* [87] enterotoxins can serve as examples. The field of diarrheas produced by the enterotoxins would not be well reviewed without mentioning a major contribution of basic research in this field. During the last cholera pandemic a break through was made in the rehydration therapy by the administration of oral fluids, containing glucose [88, 89]. This was due to studies proving the coupled sodium and glucose transport in the small intestine. Glucose accelerates the absorption of solutes and water [90, 91]. Phillips then suggested that this would allow oral rehydration of cholera [92]. Indeed the benefits are such that the method is now generally recommended by WHO [93] and has outstanding possibilities in husbandry in combating neonatal calf diarrhea [77].

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