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ENTEROTOXINS FROM GRAM-NEGATIVE BACTERIA RELEVANT FOR VETERINARY MEDICINE

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ABSTRACT

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The chemistry, mechanism of action, assay methods, pharmacology, and prevention and treatment of diarrhoea due to toxins of gram-negative microbes are discussed. Other virulence factors are mentioned briefly. Special emphasis is placed on non-specific treatment by oral rehydration.

INTRODUCTION

Diarrhoea of the newborn and infants is of serious concern in human medicine mainly in developing countries. Diarrhoea of newborn and young animals results in serious economic losses in husbandry (House, 1978; Colloquim, 1978). It is therefore important to follow the rapid development of knowledge about etiology, pathogenesis, prevention and treatment of this syndrome. During the last decade evidence has accumulated for the multietiology of diarrhoea of humans and domestic animals.

Though coronavirus has long been known to induce gastroenteritis in pigs, it is now established as the most serious enteric infection in calves and other animals (Mebus, 1978; Storz and Doughri, 1978). Ever since the finding by Mebus and co-workers (Mebus et al., 1969) of rotavirus as the cause of calf diarrhoea, further viruses have been discovered as etiological agents of diarrhoea in human and domestic animals, mainly infants and neonates. Rotavirus is apparently the most frequent pathogen in human infants (Anonymous, 1975). The importance of rotavirus for animal husbandry is also enormous and numerous symposia are devoted to the role of viruses in diarrhoea (see Colloquium, 1978). Nevertheless, at least in some herds, Escherichia coli is the most important cause of morbidity and mortality of young animals (Wohlgemuth, 1977).

This review is concerned only with enterotoxins which produce diarrhoea and, in principle, only with those which cause fluid accumulation in the small intestine and consequently diarrhoea. There are few fields concerning infectious diseases where knowledge has accumulated as rapidly as in the elucidation of diarrhoea produced

by enterotoxins of gram-negative micro-organisms. As this review is intended to focus on veterinary medicine, the human aspects and mainly cholera enterotoxin will be stressed only where they have impact on the relevant questions.As Escherichia coli enterotoxins are of primary concern the main emphasis will be on them.

ESCHERICHIA COLI ENTEROTOXINS

For many years Escherichia coli has been considered as the cause of neonatal enteric infection in infants and domestic animals. The classic concept presumes that specific, so-called enteropathogenic O serogroups of Escherichia coli, different for each species, are the cause of the majority of neonatal enteric infections. Until 10 years ago this was generally accepted (Barnum et al., 1967; Sedlák and Riesche, 1969). It is the outstanding merit of Smith and Halls to have discovered the enterotoxic activities, the basis of all the progress achieved during recent years. In their papers, Smith and Halls (1967 a,b) published a detailed analysis of infections by various Escherichia coli strains in different species of domestic animals, causing diarrhoea mainly in calves and pigs. They demonstrated that not only the E. coli bacterial cultures but also the filtrates from them produced fluid accumulation in homologous ligated loops of the small intestine. Endotoxin from these cultures did not cause fluid accumulation.

Thus the authors came to the conclusion that Escherichia coli strains which produce diarrhoea must have the ability to proliferate in the small intestine and must produce an enterotoxin which is responsible for fluid accumulation. They discerned a heat-stable (ST) and heat-labile (LT) enterotoxin, responsible for fluid accumulation. Even more important, the same group (Smith and Halls, 1968; Smith and Linggood, 1971) demonstrated that the enterotoxin production depends on the presence of the transferable extrachromosal plasmid ent^+. All their findings have since been confirmed many times.

Heat-labile Escherichia coli enterotoxin (LT)

Purification

Numerous earlier attempts to establish the molecular weight and nature of the heat-labile enterotoxin gave very different results (Söderlind et al., 1974; Konowalchuk et al., 1978).Only Gill et al. (1976) found a molecular weight of 23 000 which corresponds to the subunit A of LT as now established. Within the last 2 years several groups have achieved marked progress in the purification and clarification of the structure of LT.

(1) The Dorner group

Dorner and coworkers (1979) synthesized Escherichia coli LT in a cell-free medium.

They used the plasmid P307 (molecular weight 65 x 10^6) which was first described in the E. coli strain P307 isolated from an outbreak of porcine diarrhoea. The plasmid was transferred to a plasmid-free laboratory strain and used as the source of ent^+ plasmid DNA. DNA of the drug resistant plasmid NR1 isolated from E. coli CR34/R12 was used as a control template. LT was synthesized in a complex cell-free medium. To synthesize labelled proteins, tritiated amino acids were added to the cell-free mixture which contained plasmid DNA. The synthesized product was positive in the Chinese hamster ovary cell assay (Guerrant et al., 1974). The effect could be antagonized by antiserum against purified cholera toxin (anticholeragen) and by heterologous antitoxin prepared in rabbits against semipurified toxin from the enterotoxigenic E. coli strain P263 (Dorner et al., 1976). The active product was heat-labile and activated cyclic adenosine monophosphate (cAMP) in a dose-dependent way. For the production of LT the presence of spermidine was necessary. Heating abolished the activity of the cell-free preparation.

For purification the technique of specific immune precipitation was used. The labelled toxin was complexed with anticholeragen and adsorbed to the Cowan I strain of Staphylococcus aureus. Almost all of the enterotoxin was eluted with a combination of sodium dodecyl sulphate at high temperature. Polyacrylamide gel filtration followed.

When gel slices were assayed for the ability to stimulate adenylate-cyclase activity in pigeon erythrocyte ghosts, two peaks were found, one corresponding to a product of molecular weight 26 000, the other, when present, of 23 000. Detection of radiolabelled protein by fluorography and scintillation counting of gel slices revealed three polypeptides, two corresponding to the previously found 26 000 and 23 000, the third with a molecular weight of 11 500, identical to that of the cholera enterotoxin subunit B.

From the experiments it may be concluded that heat-labile enterotoxin synthesized in cell-free medium has a subunit structure analogous to cholera toxin, i.e. an active subunit A - 23 - 26 000 associated with 3-4 subunits B each 11 500 daltons. Thus the toxin has similar biological and immunological properties to cholera enterotoxin.

(2) The Falkow group

The group (So et al., 1978) isolated the gene for LT production using recombinant DNA technology. They were able to reduce the LT gene-containing fragment and linked it to a plasmid. The new plasmid EWD 299 encoded for LT. They proceeded with their studies by means of minicell plasmid encoded proteins. By further processing they detected two proteins, one with a molecular weight of 25 000, the other 11 500. Further analysis revealed that LT was composed of at least two distinct proteins, one of which was immunologically related to the B subunit of cholera toxin. LT was

intracellularly present as a multimetric toxin. The A and B subunits of cholera-toxin are linked by a disulphide 5 500 dalton binding structure. The authors could not find evidence for such a bond in the 25 500 dalton part of LT. But they have enzymatic and genetic evidence that the 25 000 dalton part stimulates adenylate-cyclase (Dallas and Falkow, 1979).

(3) The Finkelstein group

Recently a step which facilitates the purification of LT was discovered (Cléments and Finkelstein, 1979; Finkelstein and Cléments, 1979). They exploited the property of adherence to gel filtration matrices containing agarose with consecutive elution by galactose. The purified toxin from a strain of E. coli of human origin gave three bands – 28 000 daltons, 24 000 daltons (active in biological assays) and a 11 500 fragment. Thus, their results agree with those of Dorner and coworkers that LT consists of two subunits A and B. They are immunologically distinct from each other but are related to the corresponding subunits of choleragen. They found slight differences among the products depending on their sources. The subunits of cholera-toxin and LT are very similar. But only 15 of the 20 N-terminal amino acids are identical.

(4) The Robertson group

The molecular weight of LT was found to be 74 000, consisting of two subunits A and B (Robertson et al., 1979). The A subunit had a molecular weight of 28 200, the B subunit 11 500. The A subunit was found to be different from the cholera toxin A unit, whereas the B subunit was identical to that of the corresponding cholera subunit. The Escherichia coli strains used for purification were of human and porcine origin (Robertson et al., 1979).

It is thus clear that the LT has a similar structure to cholera enterotoxin, with many similarities but some differences in amino acid composition. The complete analysis of LT and comparison with cholera enterotoxin can thus be expected in the near future.

Mechanism of action

Like cholera toxin, heat-labile Escherichia coli enterotoxin stimulates the activity of adenylate-cyclase in the mucosa of the small intestine (Field, 1971; Sharp and Hynie, 1971). This was found for Escherichia coli LT filtrates of human (Evans et al., 1972) and calf (Hynie et al., 1974) origin. The process of adenylate-cyclase activation requires NAD and ADP (Gill et al., 1976). Purified low molecular LT (molecular weight 20 000, possibly the subunit A of LT?) catalyses the hydrolysis of NAD to ADP ribose and nicotinamide. LT also catalyses the transfer of the ADP ribose moiety to arginine (Moss and Richardson, 1978). Thus LT has both NAD-glyco-hydrolase and ADP-ribosyl-transferase activities. The group described earlier the

same type of activity for choleragen (Moss and Vaughan, 1977). Immunological cross reactivity between cholera enterotoxin and LT is indeed well established (Holmgren et al., 1973; Smith and Sack, 1973 and many others). Nevertheless, Moss and Richardson (1978) found different requirements for processing to achieve the optimal enzymatic activity. Whereas choleragen activity was enhanced by increasing the potassium phosphate concentration, or by sodium acetate, both inhibited LT activity. With Tris (Cl^-) pH 7.5 the opposite was true. Thus, in spite of the great similarity, there are also some differences between the two toxins. Differences in the amino acid composition together with similarities have already been described for the A units of both toxins (Finkelstein and Clements, 1979). Moreover, the Finkelstein group (Honda and Finkelstein, 1979) have now described a Vibrio cholerae mutant producing an enterotoxin without the A portion of the cholera enterotoxin. It lacks the capacity to stimulate adenylate-cyclase.

The activation of adenylate-cyclase by Escherichia coli LT leads to an increase of cAMP in the mucosal cells of the small intestine. This increase alters the intestinal transport in two ways. It inhibits a coupled influx for Na^+ and Cl^- at the luminal border, thus reducing the absorption of NaCl and water. This is probably effected by the villus cells. The second mechanism leads to active secretion of anion and Na^+ into the lumen; this probably takes place in the crypt cells. Thus LT, like cholera toxin, induces preponderant net fluxes to the luminal side, hypersecretion, and diarrhoea (Field, 1976; Moon, 1978).

Assays of Escherichia coli heat-labile enterotoxin

Originally LT was tested by its capacity to cause fluid accumulation in the small intestine. Later suitable cell culture assays were developed. Since the purified products are available, serological methods have been introduced. The various methods will be briefly discussed.

(1) In vivo assay in ligated intestinal loops

These methods derive from the biological assay of cholera toxin. Cholera toxin introduced into ligated loops of the small intestine of rabbits produces fluid accumulation (De and Chatterjee,1953; Burrows and Musteikis, 1966). While homologous intestinal loops for enterotoxins derived from enterotoxigenic Escherichia coli (ETEC) are the most responsive, it could be demonstrated that the ileal loop of rabbits is suitable for the assay. Escherichia coli filtrates of human and animal origin (Moon et al., 1970, 1971; Evans et al., 1972) cause fluid accumulation in the ligated loops. The dynamic of fluid accumulation differs for LT and ST. The interval of 18 h between LT-containing filtrate administration and fluid accumulation was found optimal for the assay.

(2) Vascular permeability assay

For the assay of enterotoxic E. coli filtrates a method known to pharmacologists for assay of changed local vascular permeability was introduced (Evans et al., 1973a). The filtrates are injected into rabbits intradermally. Following a suitable period of time, Evans blue is injected intravenously for the detection of vascular permeability changes.

(3) A method for the assay of LT has been developed on adrenal cell monolayers (Donta et al., 1974). The toxin induces 3-ketosteroid production. Marked morphological changes of the cell (rounding) are observed and evaluated. An analogous method works with Chinese hamster ovary cells (Guerrant et al., 1974). In this case elongation of the cells is evaluated.

The most recent assay of LT in cell cultures has been described by Stavric and coworkers (1978). They use Vero cells from the African green monkey kidney. The cells react to LT and to cholera toxin by a dose-dependent increase of cAMP which precedes the morphological changes of the cells. The affected cells are enlarged, thick-walled and refractile. The authors have found this method more sensitive than the assay on adrenal cells but less sensitive than assays on Chinese hamster ovary cells. The Vero cells do not respond to ST. However, filtrates of some E. coli strains have cytotoxic effects on Vero cells and do not induce fluid accumulation in rabbit ileal loops (Konowalchuk et al., 1978).

(4) Various serological methods have been developed recently

(a) A haemagglutination test (Evans and Evans, 1977).

Erythrocytes, from species suitable for the assay, sensitised with LT exhibit passive immune haemolysis. The test is simple, requiring only antiserum to LT. Haemolysis is measured by Spectrophotometry. The Evans group developed this test for LT from strains of human origin. Recent information indicates that this test is unsuitable for LT identification produced by strains of porcine and food origin (Serafim et al., 1979). The test was less sensitive than the Y-1 adrenal cell test even for LT of E. coli strains of human origin. In the Y-1 adrenal cell test LT of E. coli strains isolated from the different species gave positive results.

(b) A solid phase radioimmunoassay has been developed for the detection of Escherichia coli LT (Greenberg et al., 1977). As LT was then not yet available in purified form, pure cholera toxin was used for the cross reaction of antiserum to LT. Solid phase immunoassay was also used by Ceska et al. (1978).

(c) Enzyme-linked immunosorbent assay (ELISA) has been elaborated for the detection of LT (Yolken et al., 1977). This assay is again based on immunological similarity between Vibrio cholerae toxin and heat-labile E. coli enterotoxin. Instead of isotope-labelled reagents as in radioimmunoassay, it utilizes enzyme (alkaline phosphatase)-labelled reagents.

(d) Swedish authors (Svennerholm and Holmgren, 1978) have used the specific binding of LT to polystyrene-adsorbed G_{M1} ganglioside with subsequent enzyme (alkaline phosphatase) immunological demonstration of the bound toxin. They consider the ELISA procedure as comparatively simple, and found excellent agreement with biological LT assays on Y-1 adrenal cells. The recent purification of LT will undoubtedly further facilitate the development of quantitative identification of Escherichia coli heat-labile enterotoxin.

(e) Burgess and coworkers (1978) have recently recommended the oral administration of enterotoxic filtrates to infant rabbits to establish enterotoxic activity in otherwise the same way as the method of Dean and coworkers (1972, see below) does for the thermostable product.

Heat-stable Escherichia coli enterotoxin (ST)

Purification

ST was originally discovered by Smith and Halls (1967). It is firmly established that the genes for the production of this toxin are plasmid-bound like those of LT (Gyles et al., 1974; Wachsmuth et al., 1976). More precise knowledge about the nature of ST was gained only recently.

(1) Alderete and Robertson (1977a) used a defined medium. Thus ST production by strains of porcine and bovine origin containing the ent^+ plasmid became more effective. The better toxin production was confined to ST and not to LT.

The production of ST can be repressed by the addition of D-glucose, d-glucose-acetate, L-arabinose and β-galactosidase. This inhibition of ST synthesis can be overcome by the addition of cAMP (Alderete and Robertson, 1977b). The supernatant of an E. coli strain of porcine origin was purified (Alderete and Robertson, 1978) by using several steps: ultrafiltration, acetone fractionation of ultrafiltration retentates, chloroform-methanol extraction, preparative electrophoresis, ion-exchange chromatography on DEAE agarose, gel filtration on Bio-gel P-10. The molecular weight determined by two different methods was 4420-4425. Amino acid analysis, representing 47 residues, gave a calculated molecular weight of 5100. No trace of lipids or nucleic acids was found, but there was a positive reaction for carbohydrate. The purified ST has a characteristic UV absorption spectrum at 270 nm. The biological activity remains intact after heating to 100° for 30 min. The product resists various chemical treatments and can tolerate wide changes of pH from 1.0 to 9.0. In contrast to older views about the nonantigenicity of ST, it is possible to antagonise the biological activity by antisera (Alderete and Robertson, 1978). But apparently ST is a poor antigen.

(2) Kapitany et al. (1979) purified heat-stable E. coli enterotoxin from a strain of bovine origin. Its properties were roughly similar to those of the purified ST toxin of Alderete and Robertson. ST from an Escherichia coli strain isolated from the faeces of a calf differed in several points from the product of Alderete and Robertson (1978). The crude products of both groups were heat-stable, the purified toxin of bovine origin was not heat-stable (Kapitany et al., 1979). The amino acid composition and yield of the ST purified by Alderete and Robertson was different.

(3) Nalin and coworkers (1978) have isolated ST and LT from Escherichia coli strains of human origin. These induced secretion in dog intestinal loops. However, they remained negative in the infant mouse assay even after concentration of the supernatant. The authors suppose that this might indicate a different ST. This hypothesis certainly needs further confirmation.

(4) Incongruences between different assay methods (infant mouse, ligated rabbit and piglet loops) have led another group of authors (Burgess et al., 1978) to the conclusion that there exist two different ST toxins. One is methanol-soluble, partly heat-stable, active in neonatal piglets. The other is methanol-insoluble, active in weaned pigs and rabbit intestinal loops but inactive in the suckling mouse test.

Thus further research is needed before it can be firmly established whether only one or more E. coli ST are produced and if and how much they differ between species.

Mechanism of action

There is general agreement that the ST is different from the E. coli heat-labile product (Jack and Wu, 1974; Alderete and Robertson, 1978). Unlike LT it does not stimulate adenylate cyclase with a following increase of cAMP in the intestinal mucosa (Kantor, 1975; Hamilton et al., 1978; Hynie and Rašková, unpublished). Several groups now agree that ST stimulates cyclic guanylate cyclase with resulting accumulation of cyclic guanosine 5'-monophosphate (cGMP). The guanylate cyclase stimulating capacity has been demonstrated with crude and purified ST from E. coli of human, bovine and pig origin. The increase in cGMP is rapid and precedes the increase of intestinal secretion. Exogenously administered cyclic 8-Br-GMP mimics the fluid secretion induced by ST.

From all published reports it may be concluded that Escherichia coli heat-stable enterotoxin causes intestinal secretion by increasing intestinal cGMP. It is also noteworthy that apparently the binding of ST to the intestinal mucosa is not irreversible (Field et al., 1978; Hughes et al., 1978; Newsome et al., 1978; Gianella and Drake, 1979). The fluid accumulation is due to increased secretion by the intestinal epithelium and not to increased capillary or epithelial permeability

(Presnell et al., 1979).

Bioassays

(1) Originally ST was discovered using the ligated homologous small intestine loop (Smith and Halls, 1967). Within the first years after the discovery of Escherichia coli toxins, fluid accumulation of ST in homologous or rabbit ileal loops was used (Moon et al., 1970; Evans et al., 1973) at a 6-h interval after administration.

(2) Infant mouse assay

With slight modifications this test is used by most investigators (Dean et al., 1972). The investigated product is injected into the stomach of 2- to 4-day-old mice with or without dye. Three or four mice are used per product and dilution. After 3-4 h the animals are killed, the whole intestine is removed and weighed and so is the remaining body. The intestine to remaining body ratio is calculated. In general, ratios greather than or equal to 0.085 are considered as positive. Moon and coworkers (1978) recommend performing the test at 37° using diarrhoea as the index of response. They found that, depending on age and ambient temperature, the ratio of intestine : body weight might lead to false results. The usefulness of the mouse test was confirmed in several comparative studies with the intestinal loop test (Whipp et al., 1975; Moon et al., 1976; Sivaswamy and Gyles, 1976; Schoenaers et al., 1978). Olsson and Söderlind (1980) find negative results of limited value for ST of porcine origin. The age of infant mice is important. ST produces fluid accumulation only in mice younger than 16 days (Franceschi et al., 1980). A simplified preparation of E. coli filtrates for the mouse test has been proposed by Gomes et al. (1979). Filter paper is wetted by E. coli merthiolated cultures. Up to 2 months after the procedure, the mouse test may be used after elution from the the filter paper. The test recommended for developing countries for humans might be useful in veterinary practice when a massive investigation of LT presence is necessary.

(3) Dog loop assay (Nalin et al., 1974)

This test uses small intestine loops of dogs. Concentrated ST filtrates have to be used and net absorption is measured. ST activity is observed within 20 min after administration whereas LT activity appears after a lag period of 4-6 h. This test is elaborate and thus suitable where more accurate results are needed.

(4) The perfusion of the rat jejenum in vivo

This method is advocated by Klipstein and coworkers (1976) as more relevant for identifying toxin-producing strains for both heat-labile and heat-stable toxins not only in strains of Escherichia coli but also in Klebsiella and Enterobacter

cloacae. The toxins induce net water secretion into the perfused intestine (Klipstein et al., 1979). So far only material of human origin has been tested.

Common and miscellaneous features of LT and ST

Comparison of various assays

A comparison of several assay tests for filtrates of E. coli, laboratory and wild strains producing LT and ST, was performed by Kétyi and coworkers (1978). In their hands the vascular permeability test was the most sensitive, followed by the suckling mouse test. Increased vascular permeability is induced by many pharmacologically active compounds, thus some caution is advisable in assessing whether the increased vascular permeability induced by a crude E. coli filtrate is due only to enterotoxin.

Clinical course of E. coli enterotoxin induced diarrhoea

The frequency of ST and LT producing E. coli strains varies with species and ecological conditions. Recent findings (Moon et al., 1980) indicate that E. coli strains from piglets producing K 88, produce LT, those with other colonising factors in general produce ST. Many strains produce both toxins. Diarrhoea has always the same consequences. Loss of water and electrolytes leads, depending on severity, to clinical signs of dehydration, acidosis, dehydration shock and death of the animal (Tennant et al., 1972). The clinical course is the same whether the causative reagents are E. coli enterotoxins, other enterotoxins of gram-negative micro-organisms or viruses. The age factor, however, is very important. The younger the animal, the shorter the interval between the onset of diarrhoea and manifest signs of dehydration (Rašková et al., 1976). Unless the dehydration is corrected immediately, death may occur within hours.

Pharmacology

While the biochemical side of enterotoxin activity is constantly at the center of interest, less attention has been paid to changes in motility of the small intestine as an important factor in the development of diarrhoea. Two groups are interested in this field.

With E. coli strains and partially purified and desalted culture filtrates Metz and Ohgke (1976) produced impaired rhythmicity in rabbit ileal loops. The effect persisted after heating, which points to ST production.

The other group (Mathias et al., 1976; Burns et al., 1978) found changed myoelectric activity in distal rabbit ileal loops (a migrating acting potential complex). This could be elicited by cholera toxin, and culture filtrates from ST and LT producing E. coli strains. The changes occur at random and this is in agreement with the mechanical tracings of Metz and Ohgke.

Interesting are the findings of Pesti and Gordon (1978). They tested ST filtrates on various smooth muscle preparations. The filtrates per se were not especially active. However, they did clearly antagonise alpha-adrenergic effects on various preparations. This was clearest on isolated rabbit aorta strips. The ST filtrates behaved like the alpha blocker phentolamine. The authors claim that antagonism to beta-blocking agents was irregular and that the results were different from endotoxin and choleratoxin. Results of such experiments, however, are not included. An increase of catecholamine content in plasma, urine and the intestinal wall of rabbits after intravenous injection of choleratoxin was observed and connected with fluid accumulation in the intestine (Pervuchina and Ramaeva, 1978).

The ST toxin had positive inotropic activity antagonised by propranolol in the embryonic chick heart (Hedtke et al., 1976). As purified E. coli enterotoxins become available, this type of experiment should be repeated.

Results after parenteral LT or ST administration should be evaluated with care. Choleratoxin and E. coli enterotoxins act locally in the intestine, and parenteral injection does not correspond to the natural disease (Rašková, 1976).

Prerequisites for the appearance of enterotoxin-induced diarrhoea

Escherichia coli and other gram-negative bacteria must acquire and harbour plasmids necessary for enterotoxin production. The micro-organisms must be present in the small intestine in high numbers to produce a sufficient amount of enterotoxins.

To remain in the small intestine the micro-organisms must have means of counteracting the caudal movements of peristaltics. This is achieved by specific adherence (colonising) factors. The toxin then must penetrate the membrane of the epithelial cells of the small intestine to induce on the inner side of the membrane the above-explained changes of adenylate and guanylate cyclase activity. The increase of water and solute secretion leads to a preponderance of fluxes into the lumen of the gut.

To penetrate the membrane barrier a number of toxins, including Escherichia coli enterotoxin, have to bind to specific membrane receptors.

Colonising (adherence) factors

The state of knowledge concerning adherence factors until 1976 has been reviewed (Smith, 1977). Colonising factors are pili (fimbriae) or pili-like structures, frequently of filamentous character. They protrude from the surface of the microbe. So far three colonising factors are known, which adhere to the mucosa of pigs, lambs and calves. They are plasmid mediated proteins. K 99 is a colonising factor of E. coli in calves, lambs and pigs (Isaacson, 1978), K 88 plays a role in pigs (Smith, 1977), P 987 is found in porcine E. coli strains (Moon et al., 1976; Isaacson et al., 1978). Two colonising factors (CFA/1, CFA/2) have been found in human strains

(Evans et al., 1977, 1978).

K 88 is a pilus-like structure, a protein of 25 000 daltons (Moon et al., 1979). K 99 is also a pilus-like structure, a protein. Two subunits of 22 500 and 29 000 daltons were found and there is a tendency to filamentous aggregation (Isaacson, 1978). Apparently the cell-free K 99 antigen is a glycoprotein with a terminal-linked galactose moiety important for binding (Morris et al., 1977, 1978). The human colonising factors CFA/1 and CFA/2 are proteins, of molecular weight 23 000. The human factors also colonise rabbit small intestine (Evans et al., 1978, 1979). The adherence of K 88, K 99 and P 987 can be assayed in vitro on porcine epithelial cells (Jones and Rutter, 1974) and by haemagglutination tests (Moon et al., 1979).

A haemagglutination test for the assay of the CFA factors has been described (Evans et al., 1979). It is necessary to remember that E. coli also possesses so-called common pili or type 1. The colonising and other pili are distinguishable by the difference in haemagglutination patterns. The colonising factors exhibit mannose-resistant haemagglutination in erythrocytes of different species, the pili 1 haemagglutination is mannose-sensitive.

According to the latest views (Moon et al., 1980), many E. coli strains from neonatal pigs produce one of the three colonising factors, and yet others do not, and still adhere to the intestinal mucosa and produce enterotoxins. From this paper it also seems to be established that a second heat-stable enterotoxin (STb) exists. This is detectable only in the homologous small intestine loop assay. The latest report from the Evans group (1980) also reported variations in sensitivity to erythrocytes of different species, due to some antigen diversity of the pili.

Interesting are the results of Thorne et al. (1979). They demonstrated adherence to human buccal epithelia by enterotoxic E. coli strains of human origin. This method certainly deserves repetition with buccal cells homologous with the species from which the E. coli strains are isolated.

Hydrophobic interaction chromatography has been described as a suitable method of screening for adhesion factors (Smyth et al., 1978). The mechanism of adherence could be firstly attraction by electrostatic forces and then binding to specific receptors on the membrane (Isaacson, 1978).

The K 88 colonising factor is adhesive only in some piglets. The presence or absence of the specific receptor on the epithelial cell is inherited in a simple Mendelian way (Sellwood, 1979). Adherence, i.e. presence of the receptor, is dominant. Homozygous dominants and heterozygotes possess the receptor whereas in the homozygous recessives it is absent. These conclusions were confirmed in a natural outbreak of enterotoxic diarrhoea in piglets. In litters from homozygous recessive parents diarrhoea did not occur. Piglets from susceptible dams were also not affected, presumably being protected by specific IgA antibody obtained from the colostrum. The real danger comes from susceptible boars mated with resistant sows. Thus the use of resistant boars could lower the danger of K 88 adherence. This would prevent

diarrhoea in spite of the presence of plasmid for enterotoxin production in the infecting strain of E. coli. As the colonisation in the distal ileum of unsuckled calves proceeds 3 h post partum (Pearson and Logan, 1979), the importance of early administration of sufficient quantities of the secretory IgA-containing colostrum is evident. This field is rapidly progressing and would deserve a separate review.

Binding of E. coli enterotoxin to membranes

Adherence substantiates sufficient enterotoxin production by the E. coli cells harbouring the relevant plasmid. For choleratoxin it has been firmly established (Moss and Richardson, 1978; Sattler et al., 1978) that the toxin and its B subunit bind 4 molecules of monosialogangliotetraite, the oligosaccharide moiety of the ganglioside G_{M1}, per molecule of toxin protein. The toxin complex undergoes conformational changes that promote dissociation and entry of the A subunit into the plasma membrane (Fishman and Brady, 1976).

Moss and coworkers (1976) have demonstrated for choleragen and recently (1979) for E. coli LT that fixation occurs in the same way. E. coli enterotoxin did not induce an increased activity of adenylate cyclase in a fibroblast cell culture lacking ganglioside. When the ganglioside G_{M1} was added to the medium and incorporated in the membrane after the addition of choleratoxin or LT, adenylate cyclase activation appeared. It was also established that sialidase converts other gangliosides to G_{M1}.

Unmasking of the G_{M1} receptor sites by choleratoxin has been found in intestinal mucosa homogenates by Gascyone and Van Heyningen (1979). In general the stronger diarrhoeic effect of choleratoxin might be due to the receptor unmasking effect by the sialidase of V. cholerae. The binding to G_{M1} apparently takes place in any cell membranes where G_{M1} is present. This was demonstrated for adrenergic, sensory and motor neuron nerve endings (Stoeckel et al., 1977).

Tayot has used the fixation to G_{M1} to purify choleratoxin (1979). He coupled G_{M1} to silica beads with DEAE dextran (Spherosil). Crude filtrates are filtered through the particles and a desorption of choleratoxin is achieved at pH 2.8. The same author advocates the coupling of high amounts of G_{M1} with sheep erythrocytes to be used for a haemagglutination test. It would be interesting to use the same approach for E. coli LT.

OTHER ENTEROTOXINS

The rabbit ileal loop method is so far the preferred method for establishing enterotoxic activity of cell-free filtrates of various micro-organisms.

Since the discovery of enterotoxic activity of some Clostridium perfringens strains (Duncan and Strong, 1969) the importance of Clostridium perfringens-induced diarrhoea in human and veterinary medicine has become apparent (Lozano et al., 1970).

The enterotoxic is pathogenic for many species of domestic animals, and within the context of this review its occurrence in cattle, sheep and chicken is most important (Niilo, 1978). The fluid accumulation is also demonstrable in mouse ligated intestine (Yamamoto et al., 1979).

Attempts to purify the toxin have been made and so far the enterotoxin from Clostridium perfringens type A appears to consist of one polypeptide chain, 34 000 daltons (Granum and Skjelkvåle, 1977).

Mechanism of action: Like E. coli enterotoxins, this toxin induces in the ileum a preponderance of net secretion. This concerns mainly water, sodium and chloride ions, although their absorption remains normal (McDonel, 1979). Glucose is still absorbed but at a lowered rate. Activation of adenylate cyclase was not observed (McDonel, 1979). The toxin causes desquamation of intestinal cells (McDonel, 1979). Apparently, in agreement with electron microscopic studies (McDonel and McClane, 1979) the brush border (microvillus membrane) of the villus tip of the epithelial cell is the primary site of action of this enterotoxin.

The effect of the toxin on membrane permeability and amino acid transport was studied in primary cultures of adult rat hepatocytes (Giger and Pariza, 1980). The toxin induces a decrease of -aminoisobutyric acid. This is correlated with a rapid increase in intracellular Na^+, apparently a sign of membrane damage. Later, the enterotoxin increases the exodus of L-glucose, 3-O-methylglucose and α-aminoisobutyric acid from pre-loaded cells, indicating that the membrane undergoes progressive damage. Rapid release of lactate dehydrogenase from isolated hepatocytes was observed, another indication of membrane damage (Skjelkvåle et al., 1980).

Clostridium perfringens enterotoxin binds rapidly to intestinal or Vero cells. This binding to receptors is a prerequisite for the action of the C. perfringens enterotoxin (McDonel and McClane, 1979). There are several biological assays to test C. perfringens enterotoxin:

(1) The rabbit ileal loop (Duncan and Strong, 1969).

(2) The mouse ileal loop (Yamamoto et al., 1979).

(3) The increased vascular permeability test (Stark and Duncan, 1972).

The Clostridium enterotoxin story is now even more fascinating. Diarrhoea is a frequent side effect of antibiotic administration. Clostridium difficile has been implicated as the cause of this diarrhoea and the serious pseudomembranous colitis that results (Bartlett et al., 1978). The role of C. difficile toxin has since been confirmed from various sides (George et al., 1977). Bartlett and coworkers (1978) have partly purified the toxin. This product is heat and acid labile, sensitive to trypsin, and has a molecular weight of 240 000. Colitis caused by antibiotics was first described in hamsters (Small, 1968). Enterocolitis is also easily induced by some antibiotics in rabbits (Katz et al., 1978). C. sordellii is of marked pathogenicity for rabbits and guinea pigs. In humans C. sordellii also plays a role, but apparently C. difficile is more important (Loeschke, 1980). Diarrhoea

and pseudomembranous colitis caused by Clostridium toxins have been confirmed for hamsters and guinea pigs (Larson et al., 1979).

C. difficile alone or in connection with other clostridia is the cause of spontaneous neonatal diarrhoea in infant hares (Dabard et al., 1979). Undoubtedly more attention should be paid in husbandry to diarrhoeas and colitis induced by antibiotics.

Fluid and electrolyte accumulation in the rabbit ileal loop is now reported for an increasing number of filtrates of bacterial origin. Shigella dysenteriae enterotoxin (Keusch et al., 1972) is interesting only for human medicine. It does not stimulate adenylate cyclase in the regular manner of E. coli LT (Charney et al., 1976). Enterotoxin-producing Aeromonas hydrophila, Plesiomonas shigelloides and non-agglutinating vibrios (NAG) have been isolated from smaller numbers of cows, calves, pigs and sheep (Karolček et al., 1979). The crude culture filtrates and a protein fraction of 60 000 daltons from NAG vibrios stimulated secretion in the rabbit ileal loop (Čižnár et al., 1977, 1979). Dobrescu (1979) described an LT-like enterotoxin from Aeromonas hydrophila filtrates of porcine origin. An increase of cAMP in mucosal cells was also found. Positive reactions in Y-1 and CHO cell cultures were observed (Ljungh and Wadström, 1979).

Campylobacter also deserves mention. Venereal campylobacteriosis is a current problem (Roberts, 1979). Even 50 years ago the micro-organism was incriminated in cattle diarrhoea (Jones and Little, 1931). The improved isolation from cattle faeces might renew interest in research. Chickens are frequently incriminated as the source of human infection (Karmali and Fleming, 1979). Yersinia enterocolitica frequently produces a heat-stable enterotoxin (Boyce et al., 1979) and this is produced by about 50% of strains isolated from animals (Pai et al., 1978; Kapperund et al., 1980). The micro-organism is the etiological factor for numerous human diarrhoeas and certainly deserves attention in veterinary medicine. Attention has to be paid also to the so-called opportunistic micro-organisms which in the old times were supposed to be saprophytes. B. cereus is a good example. Some strains of this micro-organism produce an enterotoxin with necrotic properties. As B. cereus is the cause of numerous food poisonings in man from milk and other foods (Turnbull et al., 1977), it should be studied in animal husbandry too. Other examples could be given. Klebsiella pneumoniae, Citrobacter (Rašková et al., 1975; Guerrant et al., 1976), Pseudomonas aeruginosa (Yamamoto et al., 1980) and so on. The number of micro-organisms known to produce enterotoxins will undoubtedly increase in the future and much further research is needed in the field.

ATTEMPTS AT SPECIFIC PREVENTION OF ENTEROTOXIGENIC E. COLI-INDUCED DIARRHOEA

In cattle and pig production economic losses from diarrhoea are very serious (House, 1978). Infection occurs mainly in the earliest postnatal period. In calves

and pigs specific prevention is complicated. In both species transplacental immunity is almost nil and passive immunity has to be acquired via the colostrum (Barnum, 1971; Fey, 1971). To increase lactogenic immunity for the young the mother must be immunized. The original concept presumed that enteropathogenicity of E. coli depends on a small number of O serogroups which are different and specific for each species (Sedlák and Riesche, 1969; Orskov and Orskov, 1978). This concept of enteropathogenic E. coli as the preponderant cause of diarrhoea was the basis for immunization attempts with corpuscular bacterins. Favourable results were reported by some authors. But even in 1971, Gay reported that when this type of vaccine is tested in controlled field trials, the effects are dubious. Our group (Raška et al., 1978) conducted a controlled field trial with cows. The dams were given two intramuscular injections of a commercial corpuscular bacterin containing six different O serogroups. Under strictly controlled conditions (blind random allocated field study, strict adherence to a uniform protocol, adequate administration of colostrum, two injections to dams) no significant differences were found in comparison with placebo in the incidence of diarrhoea in the calves. We came to the same conclusions as Oudar et al. (1976).

The new knowledge about E. coli enterotoxins brought a change in concepts. To be effective the vaccine must stimulate antibodies to the relevant virulence factors, i.e. enterotoxins and/or adherence factors. The basis for the vaccines is in all cases the expectancy of a high concentration of the relevant secretory A immunoglobulins in the colostrum.

(a) Vaccines developed on the basis of heat-labile E. coli enterotoxin
The rat model was used to demonstrate the possibility of protecting animals against E. coli LT challenge (Klipstein and Engert, 1979). They administered small doses of partly purified polymyxin released E. coli LT. They used LT toxin alone (priming dose) and alone or with Freund's adjuvant (boosting dose). Combinations of different administration routes were used. One week after the boost, rats were challenged with LT and the fluid accumulation was compared with controls. For successful vaccination by the oral route, blocking of the gastric H_2-histamine receptors by cimetidine was necessary. LT-induced secretion was partly or completely absent in vaccinated animals.

In a controlled field trial Dobrescu and Zygraich (1978) compared morbidity (diarrhoea) in piglets. The sows were subcutaneously immunized with LT adsorbed on aluminium hydroxide 4 weeks before farrowing. One group received a second (intramammary) injection 1-4 days after farrowing. To one group a polyvalent corpuscular vaccine was applied. The last group received placebo in the same way. The combination of subcutaneous and intramammary vaccination by LT was the most effective. The highest morbidity was observed in the group given polyvalent corpuscular vaccine.

(b) The dam is vaccinated with purified colonisation factors, K 88, K 99, P 987 (Isaacson et al., 1977; Morgan et al., 1978; Acres et al., 1979; Nagy, 1980). Protection to challenge was always gained only to E. coli strains harbouring the

homologous factor. The necessity of further research is emphasized not only in veterinary medicine but in all recent WHO scientific group meetings and WHO-sponsored symposia. The importance of collaboration between human and veterinary research is also stressed (WHO reports, 1978, 1979; Finkelstein and Finkelstein, 1978). But even the most successful vaccination of the types described might bring only a partial solution. E. coli-induced diarrhoea as such has several pathogenetic mechanisms. The enterotoxin-producing E. coli are the object of this review. But there is already one complication. Vaccination so far is possible only with LT; the heat-stable toxin is a poor antigen.

Other E. coli are invasive. They induce a dysentery-like disease (Frisk et al., 1978; Wadström, 1978). Non-invasive and non-enterotoxic but diarrhoeic E. coli strains were isolated from rabbits (Takeuchi et al., 1978). There is a difference between the piliated phase of E. coli strains in vivo and a non-piliated phase in vitro (Moon et al., 1978). Nevertheless, strains of E. coli which produce diarrhoea by enterotoxin production or by an unknown mechanism are piliated, and have flagella. The invasive strains lack them (O'Hanley and Cantey, 1978).

Enterotoxin production and adherence factors are plasmid mediated. Loss of enteropathogenicity, especially of LT, has been found (Raška and Rašková, 1976; Evans et al., 1977). Since Gorbach and Khurana (1972) have challenged the classic conception of the few enteropathogenic O serotypes, the new concept of enterotoxic E. coli strains is now widely accepted. In principle, plasmid-mediated factors may enter any serotype. Reports now appear that combined LT and ST production in strains of human origin affects a comparatively small number of serotypes (Orskov and Orskov, 1978). The ST-producing strains belong to many serotypes (Merson et al., 1979). It has been pointed out previously that some serotypes hold the plasmids while others do not (Evans et al., 1977). This point probably should be kept in mind. It might be that colonisation factors also are more competitive in some serotypes, as suggested by the Evanses (1978).

E. coli enterotoxins are important even for adults as a frequent cause of traveller's diarrhoea, and this further stimulates vaccination research. The World Health Organization has developed a programme for the control of diarrhoeal disease (WHO, 1979) and collaboration in the field is important for further progress both in human and veterinary medicine.

The multietiology of diarrhoeas makes the task of specific prevention by immunization of the dam quite complicated. Good management practices, the most important of which is adequate colostrum feeding, and hygienic management measures which decrease the danger of massive infection are the more imperative the more herds are agglomerated in industrialized husbandry.

TREATMENT

In human medicine death from diarrhoeal disease including cholera can be below 1%, when contemporary knowledge is applied lege artis. The same is true for diarrhoea in domestic animals. Of course bacteraemia or septicaemia, where endotoxins play the decisive role, are not included (Rašková et al., 1976). Loss of water and solutes, with the changes in the extracellular and intravascular compartments and their consequences play the decisive role in severe diarrhoea and death by dehydration.

Rehydration

Replacement of water and electrolytes became the life-saving therapy in human cholera and other severe diarrhoeas during the 7th world cholera pandemic (WHO, 1970). Intravenous and peritoneal replacement by balanced electrolyte solution was advocated earlier in veterinary medicine by several authors (McSherry and Grinyer, 1954; Watt, 1965; Tennant et al., 1972). The procedure is life-saving but time-consuming and expensive. During the cholera pandemic older knowledge about the coupled transport of sodium and glucose by Ricklis and Quastel (1958), Schulz and Zalusky (1964), Schedl and Clifton (1964) and Crane (1965) was introduced into the therapy of cholera as oral rehydration. The efficacy is such that the World Health Organization is promoting this therapy by all means (WHO, 1976, 1978, 1979). Starting from the first publications in the field (Nalin and Cash, 1970) we gradually developed a simple oral rehydration programme for calves with mild and moderate diarrhoea (Rašková et al., 1974, 1976). To be applicable widely in the field, the administration has to be simple and inexpensive.

The crucial point is the amount of sodium, because it is desirable to give the solution ad libitum. We found the following composition to be adequate: water (drinking quality) 10 litres, sodium chloride 27 g, potassium chloride 15 g, sodium bicarbonate 26 g, glucose 200 g. The ingredients are not weighed: 2 level tablespoons (content 15 g water) of NaCl, 2 tablespoons of $NaHCO_3$, 1 tablespoon of KCl and a cup (about 300 ml) of glucose are used. The error is 10-15%. The animals drink the solution readily. All ingredients are very inexpensive. This solution, administered early after the onset of diarrhoea, gives excellent results. With persisting diarrhoea the solution is offered between feeding (the basic solution would not be favourable for the milk clotting in the abomasum). The results on many thousands of calves are excellent and our State Veterinary Service recommends the method.

Klipstein and Engert (1978) have shown in the rat model that the presence of glucose facilitates water transport from the small intestine in the presence of enterotoxins. Oral rehydration is at present the method of choice in the treatment of diarrhoea, whatever its origin (Nalin et al., 1979).

Antibiotics

Even nowadays antibiotics are widely used in humans and in animal husbandry for the prevention and treatment of diarrhoea. In large agglomerations of animals, resistance to antibiotics is so high that their therapeutic value in the treatment of diarrhoea is nil (Shull and Frederick, 1978; Raška et al., 1979). They even contribute to poorer results (Oxender et al., 1973; Rašková et al., 1976). The use of antibiotics in E. coli enterotoxin-induced diarrhoea holds another danger. In recent years reports have appeared which indicate a common transfer of antibiotic multiresistance factors and the ent^+ plasmids. This has been documented for Escherichia coli strains of pig (Gyles et al., 1977) and human origin (Echeverria et al., 1978). The adherence factors of E. coli are plasmid mediated (Smith and Linggood, 1971; Morris et al., 1977; Williams et al., 1977). Recently (Williams et al., 1978) it has been reported that an adherence factor was transferred together with resistance to several antibiotics and the production of colicin Ib plasmid. The adherence plasmid could be segregated. But all these experiments confirm the suspicions that antibiotic pressure may contribute to the dangerous spread of plasmids for enterotoxin and adherence factors in E. coli populations. Displacement of animals (trade) further aggravates the situation (Jorgensen and Sorensen, 1979).

Attempts to modify enterotoxin-induced diarrhoea by drugs

Attempts to stop secretion induced by E. coli LT and ST may be divided into three categories.

(1) Formerly it was believed that diarrhoea is mediated by prostaglandins, the rationale for administration of aspirin, and other antiflammatory drugs. Intravenous aspirin decreased the amount of fluid secretion induced by cholera toxin (Finck and Katz, 1972). The same was described for Indomethacin (Gots et al., 1974). ST-induced secretion was diminished in the infant mouse model (Madsen and Knoop, 1978). Favourable results on the same model were reported with ST and phenylbutazone (Ohgke and Wagner, 1977). All these experiments used ligated intestinal loops in experimental animals. We tested aspirin 50 mg/kg (orally) in a field trial with calves. The drug was given for 12 days. We saw no difference in the incidence and severity of diarrhoea. However, we did not measure the faeces quantitatively.

(2) Nicotinic acid diminished secretion induced by cholera toxin and prevented the increase of cAMP in the intestinal mucosa. Thus nicotinic acid could interfere with the increased cAMP level. The mechanism by which this is achieved has so far not been elucidated (Turjman et al., 1977).

(3) Chlorpromazine lowers secretion and cAMP levels in the mucosa of piglets infected with LT-producing E. coli. In field conditions Chlorpromazine shortened the duration of diarrhoea in newborn piglets. But the drug was administered simul-

taneously with trimethoprim and oral rehydration fluid (Lönnroth et al., 1979). Hence further experiments are necessary.

Recently Keusch (1979) reviewed the progress in knowledge about membrane receptors and some future preventive and therapeutic research possibilities following from the better understanding of the specific bindings on intestinal mucosa receptors. Most of our knowledge in the field comes from pharmacology. Professional pharmacology is now represented by the rapidly growing branch of immunopharmacology: competition by oral administration of specific receptors like the ganglioside G_{M1}, biochemical engineering and so on. It is questionable whether the specific toxin receptors are really specific and their original function might be autopharmacological regulation. Undoubtedly there will be rapid development along these and other lines. The vaccine development for newborns is specific. Enteric diseases, whether bacterial (non-invasive) or viral depend on IgA local immunity which is independent of systemic immunity (Pierce, 1978; Pearson and Logan, 1979; WHO, 1979). It takes some time before the immunological response of the newborn is sufficient, and thus lactogenic immunity is of primary importance. Extensive research goes on in the whole field. Hopefully we may expect that in a few years' time many problems considered here will be overcome, just as much progress as has been made since our previous review (Rašková and Raška, 1977), and so it should be in science.

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KURZFASSUNG

Rašková, H. und Raška, K., 1980. Enterotoxine von gramnegativen Bakterien in der Tiermedizin. Vet. Res. Commun., 4: 195-224 (in Englisch).

Chemie, Wirkungsmechanismen, Nachweismethoden, Pharmakologie, Prophylaxe und Behandlung von Diarrhoen, die durch Toxine gramnegativer Mikroorganismen hervorgerufen werden, werden diskutiert. Andere Virulenzfaktoren werden kurz besprochen. Besondere Aufmerksamkeit wird der unspezifischen Behandlung durch orale Elektrolytgaben gewidmet.

RESUME

Rašková, H. et Raška, K., 1980. Les enterotoxines des bactéries à gram négatif importantes en médecine vétérinaire. Vet. Res. Commun., 4: 195-224 (en anglais).

La biochimie, le mécanisme d'action, les méthodes d'études, la pharmacologie, la prévention et le traitement des diarrhées dues aux toxines des bactéries à gram négatif sont discutés. Les autres facteurs de la virulence sont brièvement mentionnés. Une attention particulière est accordée aux traitements non spécifiques, réhydratation par voie orale tout spécialement.

RIASSUNTO

Rašková, H. e Raška, K., 1980. Enterotossine di batteri gram-negativi importanti per la medicina veterinaria. Vet. Res. Commun., 4: 195-224 (in Inglese).

Vengono discussi la chimica, il meccanismo di azione, le tecniche di saggio, la farmacologia, la prevenzione e il trattamento delle forme diarroiche dovute alle tossine dei microorganismi gram-negativi. Un breve accenno viene anche fatto a proposito di altri fattori di virulenza. In particolare rilievo viene posto il trattamento aspecifico mediante reidratazione per via orale.