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SWINE AND CATTLE ENTEROTOXIGENIC *ESCHERICHIA COLI*-MEDIATED DIARRHEA. DEVELOPMENT OF THERAPIES BASED ON INHIBITION OF BACTERIA-HOST INTERACTIONS

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Enterotoxigenic *Escherichia coli* (ETEC) frequently occurs in diarrheal disease afflicting domestic animals. In this paper is summarized the research carried out over the last decade on the two important determinants of virulence that play a role in the development of the infection, namely the colonizing ability of the small intestine mediated by specific fimbrial adhesins acting as lectins and the production of enterotoxins. Recent progress in knowledge of the phenomenon led to alternative strategies of prevention and cure of enteric infection. Since bacterial recognition of mucosa surface receptors in an initial event in colonization, several approaches based on the competitive inhibition of ETEC adhesion have been developed. This review examines the following approaches: competitive colonization with non pathogenic strains, design of adhesin or toxin vaccines, receptor analog therapy and methods for *in vivo* suppression of virulence factors.

INTRODUCTION

Diarrheal disease is a major health problem and results in a heavy morbidity and mortality among young domestic animals. It is quite evident that *Escherichia coli* stands out as an important agent associated with acute diarrhea (3, 55, 94, 112, 118, 177). *E. coli* strains that produce infection may be divided into enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroadherent groups, on the basis of the mechanism of induction of disease (79). Verotoxigenic and necrotizing strains have been also isolated from diarrheic calves (32, 52, 144).

Enterotoxigenic *E. coli* (ETEC), produces one or both types of enterotoxins (131) and fimbrial adhesins which permit colonization of small bowel epithelium (19, 25, 44, 102, 129, 135). It is clear that the specific association formed between host and bacteria is complex and multifunctional. Capsular polysaccharides, lipopolysaccharides, and outer membrane proteins also play varying roles in the bacteria-host relationship (63, 140). The importance of specific adhesins and following colonization in pathogenesis is documented by experimental data obtained from infected animals (24, 36, 100, 128, 142). The occurrence, production, protein chemistry, immunology and molecular genetics of the fimbriae (or pili) have been the aim of extensive studies and reviews (29, 30, 47, 64, 71, 93, 106, 108, 109, 143). Hence, this review is restricted to those aspects related to diagnosis and therapy.

To be efficient, pathogenic bacteria must compete with endogenous flora, overcome the defense mechanisms and pass through the mucus layer to access the underlying epithelial cells. In the small intestine, the mucus layer contains multiple receptors for bacterial lectins which compete with cell receptor sites (44, 76). Thus bacteria are removed by fluid and peristalsis. In

pathological situations, the mucus may facilitate the colonization and access of adhesins and toxins to enterocytes. Furthermore, adhesin-specific receptors must be available and cell surface characteristics may account for differences among individuals in susceptibility to colonization (12, 113, 134).

Although antimicrobial methods are satisfactory for curing the majority of gastrointestinal infections *i.e.* use of oral rehydration with fluid replacement and antisecretory drugs (17, 65, 139), alternative methods have been developed. Progress in defining the molecular basis for bacteria-host interaction has provided new strategies for treatment and prevention of ETEC diarrhea (34, 56, 80, 135, 136). Accumulating knowledge on the receptor specificity of the pathogens holds the promise of developing inhibitors of bacterial adhesion for prophylactic and curative approaches to diseases.

Incidence and features of enterotoxins and fimbrial adhesins.

Enterotoxins.

ETEC strains produce two types of enterotoxins. Heat-labile toxins (LT-I and LT-II), immunologically and physicochemically related to cholera toxin (CT), are high molecular weight proteins composed by a single A subunit (Mr 25,000-28,000) and five identical B subunits (Mr 12,000) assembled in a pentameric structure. ETEC isolated from pigs and cattle produce a variant, LTp-I, whereas other types, namely LT-IIa and LT-IIb, have been found in water buffalo, cattle and human. The low molecular weight and poorly immunogenic heat-stable toxins (ST) are subdivided in ST-I type (Mr 2,000), and ST-II (Mr 5,090) both found in neonatal and weaned piglets. In contrast to K88-positive ETEC strains which all produce LT-I or both LT and ST, the bovine ETEC and fimbriae F42 or 987P positive ETEC produce only ST (94, 126) (Table 1). In addition, some ETEC strains elaborate another group of enterotoxins. Porcine strains of serogroups O101 and O138 co-express ST and *Shigella*-like toxins which together enhance their virulence (90). Furthermore, hemolysin and endotoxins are frequently associated with ETEC diarrhea.

ETEC diarrhea results from an action on the ion transport mechanism. Briefly, LT toxin acts after binding of the B subunit and translocation of the A subunit through the protein kinase C (PKC) pathway. The ST-I enterotoxin binds to the cell receptor and acts through the Ca^{2+}-calmodulin pathway (54). As indicated in Table 2, ST-I action leads to increased levels of cyclic adenosine or guanosine monophosphate (cAMP or cGMP), and changes in ion fluxes (in 131). Synergy was observed between PKC activators and ST-I (173), such interactions likely play an important role in ETEC LT, ST-mediated infection. ST-II provokes bicarbonate secretion via an unclarified mechanism that does not involve activation of adenylate or guanylate cyclase.

Detection of LT is often done by its effect on target cells. Sensitive and simple assays have been developed to examine large numbers of strains from isolates, such as coagglutination tests (123), immunoassays, enzyme-linked immunosorbent assay (ELISA). The fact that GM1 ganglioside binds LTs led to the development of GM1-ELISA (151). ST production is usually determined by bioassays such as infant mouse assay (27), and the weaned pig and rabbit intestinal loop tests to detect ST-I and ST-II respectively (131). Although useful these tests cannot be routinely used since they require animal management. Thus, they have been substituted by immunological tests, elaborated by coupling ST to a carrier protein (49, 119, 152). DNA probes for enterotoxins LT and ST are excellent tools for diagnosis of ETEC strains (87, 88, 92, 103). Gene fusion technology has facilitated the development of ELISA (57). Biotin- or digoxigenic-labelled cloned DNA fragments derived from enterotoxin genes are now proposed for clinical applications (68, 117, 159). In epidemiology, maximal information is obtained when both gene probes and ELISA are employed to determine whether a strain is toxigenic or not.

Antigenically distinct fimbrial adhesins.

Several fimbriae have been identified (Table 1). Epidemiological studies established that ETEC strains expressing K88 fimbrial adhesins are associated with diarrhea in piglets, in the 6 days after birth and the postweaning period from 3 to 9 weeks old (128). K99, 987P or F42 piliated ETEC commonly cause diarrhea in newborn piglets but they occur only infrequently in the weaned ones (78, 124, 174, 178). CS31A fimbriae is associated with K99, but it is not found in any K88 or 987P porcine ETEC (23). In newborn calves, colibacillosis is caused by ETEC strains producing K99 alone or combined to F41 and FY (F17) adhesins (24, 30, 47, 50, 138). Fimbriae F165 and CS31A were isolated from young calves and piglets (23, 39). It seems likely that other fimbrial adhesins of undetermined yet antigenic structure or non fibrillar type are emerging in atypic strains (14, 67).

Fimbriae are hydrophobic high polymers composed of identical subunits (pilins) of molecular size 18,000-32,000 and minor components. Some antigenic determinants predicted by hydrophilicity calculations (71, 85, 120) are shown in Fig. 1. The various fimbriae K88, K99, 987P, F17, F42 and CS31A are plasmid-encoded (33, 50, 85, 93, 130), whereas F41 fimbriae is chromosome-encoded (4, 95). Plasmids, harbouring 987P, K99 or F42 fimbrial genes, also contain the gene for ST-I (33, 47, 130, 137). Transposon-encoded ST-I may activate pili expression as observed for 987P production (69). In contrast, fimbriae K88 and LT are coded by two different plasmids, and the expression of K88 and ST-I genes was not found in all strains. Fimbriae F17, CS31A and F165 are encoded on a plasmid which does not confer enterotoxigenicity (23, 138).

TABLE 1. O-Serotypes associated with enterotoxins and adhesins.

ETEC isolated from	O-Serotypes	Fimbriae production	Enterotoxin activity
Piglets	8, 45, 138, 139, 141, 147,149, 157	K88 (F4)[a]	LTp-I or/ and ST-I/ST-II
Piglets	9, 20, 141,147	987P(F6)	ST-I
Piglets	8	CS1541	LT, ST-II
Piglets		F42	ST-I
Piglets, lambs, calves	8, 9, 20, 64, 101,141	K99 (F5)	ST-I
Piglets, lambs, calves	8,9, 101	K99, F41	ST-I
Calves	2, 101	FY, F17	none
Calves	9, 101	K99, F41, FY	ST-I
Calves	8, 9, 20, 23	CS31A, K99	ST-I
Calves, piglets	8, 9, 11, 15, 17, 23	CS31A or F165	none
	25, 78, 101, 115,117 141, 153	or both CS31A and F165	
Cattle	...		LT-II
Piglets	141	new type of fimbriae[b]	

Most ETEC strains belong to a limited number of O and K serogroups associated with the expression of heat labile (LT) and heat-stable (ST) enterotoxins and fimbrial adhesins (14,23,50,79,94,106,149,163,174,177,178).

[a] Fimbriae have been classified as F antigens by Ørskov (106), thus F4, F5 and F6 respectively correspond to the K88, K99, and 987P adhesins. F41, F42 have not yet been ascribed to this scheme, FY has been classified as F17 (85).

[b] Kennan and Monckton (67) propose new adhesive fimbriae associated with postweaning diarrheal disease caused by ETEC of 0141 type.

The fimbriae genetic determinants have been cloned and several structural gene clusters identified. Besides the subunit production, they code for accessory "helper" proteins which possess distinct functions in the assembly system. Biogenesis of fimbriae requires the transport of fimbrial subunits across cytoplasmic membrane, periplasm and outer membrane, the anchorage in outer membrane and finally a polymerization process at the bacteria surface (29, 108).

Most fimbriae are recognized by their ability to agglutinate a variety of erythrocytes in the presence of mannose (MRHA) (13, 20, 38, 93, 163) (Table 3). It is therefore interesting to identify and quantify the fimbriae present in ETEC directly in faeces or after isolation from intestines of diarrheic animals since this information is directly relevant to the pathogenesis aspects. When several types of fimbriae are present together on a strain, immune techniques are essential for their identification and quantitation (16, 98, 102, 106, 165). Specific monoclonal antibodies to fimbriae were produced to progressively replace conventional polyclonal antisera in diagnosis (5, 114, 161, 162, 170, 171). DNA probes were widely used to characterize fimbriae (88, 92). Furthermore, DNA hybridization offered the opportunity to test concurrently for fimbriae and toxins (68, 88, 92, 176).

LT enterotoxins and fimbrial adhesins act as lectins.

The ability of fimbrial lectins to recognize receptors is either an intrinsic property of the major fimbrial subunit itself (K99, K88 fimbriae) or mediated by a minor component (fimbriae type 1). In this case receptor-binding activity is described as tip-wise (72). Studies on bacterial binding are currently performed *in vitro* using secreted and membrane-bound glycoconjugates of various cell types and tissues such as erythrocytes, mucus, enterocytes, brush borders preparations, and epithelial cell lines (26, 66, 76, 91, 134, 166). Hydrogen bonds, hydrophobicity and surface charge characteristics are involved in adhesion (2, 61, 66, 121, 135). Specificity determination was initiated by screening for binding inhibitors among simple or complex carbohydrates. Primary structure of a number of toxins and fimbrial subunits has been partially or completely resolved, and receptor-binding sites for various lectin types identified in a few cases (Fig. 2). Comparison of structures permits us to discern, on the one hand, conserved sequences essential for the production of pili and on the other hand, highly variable regions that determine antigenic and lectins specificities (Figs. 1&2).

Figure 1.
Some potential antigenic epitopes of fimbriae.

K88		
ab,ac,ad	D^{19}-D-Y-R-Q-K	(89)
ab	Y^{213}-R-E-D-M-E-Y	(73)
ac	P^{163}-R---G-S-E-L-P-G	(9)
ac	L^{147}-S-L-F-A-D-E-G-L-S-S-I-F-Y	(158)
ad	P^{163}-T-N-V-Q-N-S-A-L-P-G	(9)
ad	F^{213}-Y-N-E-N-M-A-Y	(73)
K99	G^{41}-T-V-V-D-F-K-L^{48}; G^{69}-S-M-N-S-L-G-F^{76}; G^{82}-N-T-A-A-K-G^{88}; N^{106}-I-N-T-S-F-T^{112} F^{154}-L-V-T-Y-M^{159}	(120)
F17	S^{17}-V-T-T-E-S-K-N-L^{25}	(85)

*The factor a, common antigenic determinants has been recently separated in 7 epitopes clusters (a1 to a7), whereas the specific determinants b in b1, b2 , c, and d are unique factors (170). Region 163-173 is part of a domain with both c and d specificity. Conserved regions contain common antigenic determinants and variable clusters of the variable epitopes.

Figure 2.
Relationship between the function and structure of bacterial lectin families: active aminoacid sequences containing receptor-binding sites.

CT-B:	**K-K-A-I-E-R**^{67}
LTI B	**G**^{33}-**R**^{35}----**K-K-A-I-E-R**^{67}------**W**^{88}
K99	**K**-K-B----B-**R**^{136}
K88ab	N^{143}-**G**-E-L-**F-S-L-F-A-N**-G^{153}
K88ac	N^{143}-**G**-E-L-**L-S-L-F-A-N**-G^{153}
K88ad	N^{143}-**G**-E-L-**M-S-L-F-A-N**-G^{153}
STIp	N-T-F-**Y-C-C-E-L-C-C-N-P-A-C**-A-**G-C-Y**
STIh	N-S-S-N-**Y-C-C-E-L-C-C-N-P-A-C**-T-**G-C-Y**

Underlined amino acids are important in the receptor binding sites. Cholera toxin, LT B subunits and K99 fimbriae are three sialic acid specific lectins. In STs of bovine, porcine and human ETEC (126, 153), all the synthesized peptides containing the underlined sequences show biological activities similar to that of native forms (153). The two distinct STs, namely STp and STh of porcine and human origin respectively, differ only slightly in their primary sequences.
Jacobs et al. showed that the hemagglutination activity of K88ab and their adherence to intestinal brush border cells are inhibited by tripeptides 148-SLF and 156-AIF (62).

Sialic acid-specific lectins.

The best characterized ETEC lectins considered here are sialic acid ones. Cloning of genes for heat-labile *Vibrio cholerae* and LT toxins, and K99 subunit has shown homologies between carbohydrate recognition sequences (Fig. 2). Binding of the LT and CT is mediated by their Lys^{62}-Arg^{67} segments. Moreover, the amino acid residues Gly^{33}, Arg^{35} and Trp^{88} are also involved (165). In the same manner, Lys^{132} and Arg^{136} are essential in the binding of K99 subunits (62).

LT-Is and CT strongly bind to GM1 ganglioside Gal(β1-3)GalNAc(β1-4)[SA(α2-3)]Gal(β1-4)Glcβ1-Cer (45, 150) and to a lesser extent to GD1b ganglioside Gal(β1-3)GalNAc(β1-4[SA(α2-8)SA(α2-3)]Gal(β1-4)Glcβ1-Cer (45) (Table 4). LT-IIa binds to the same terminal sequence Gal(β1-3)GalNAc(β1-4)[(NeuAc(α2-3)]Gal with an additional contribution of a second NeuAc in GD1b and GD2. LT-IIb recognizes in GD1a and GT1b the NeuAc(α2-3)Gal(β1-4) GalNAc terminal sequence (45).

Furthermore, epidemiological studies indicate a predisposition of individuals with blood group O to be affected by CT cholera infection (58), and CT binding has been recently linked to the presence of blood group-active glycoconjugates in pig (11). Although an extrapolation is premature, the existence of common epitopes in the receptor binding sites of LTs and CT (155) (Table 3) favours the hypothesis of a relationship between blood group determinants present in glycosphingolipids and glycoproteins and susceptibility of animals to ETEC-LT mediated infection.

K99 lectin recognizes NeuGc(α2-3)Gal(β1-4)Glc-Cer (GcGM3) (141). The N-glycolyl group of the sialic acid is essential in the binding to the cell, as demonstrated by the strong decrease observed with N-acetyl or 7,9-O-acetyl derivatives (81, 104). K99 lectin binds to sialoglycosphingolipids in the following range affinity: NeuGc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glcβ1-Cer (NeuGcSPG) > NeuGc(α2-3)Gal (β1-4)Glc-Cer (NeuGc GM3) > 4-*O*-NeuGc(α2-3)Gal(β1-4)Glc-Cer >> NeuAc (α2-3)Gal(β1-4)Glc-Cer (GM3) (75, 104, 157) (Table 3). Furthermore, K99 lectin binds to mucins (82, 96). Host range of K99 piliated bacteria is determined by the occurrence of specific glycoconjugates and the individual variations in susceptibility are due, at least in part, to differences in the balance between bacteria and receptors. Resistance to K99-caused infection among adult animals (124) is due to a modification of K99 intestinal receptors by the disappearance of *N*-glycolyl groups in glycolipids of mucosa (157). On the other hand, we determined that young Large-White piglets may be distinguished in adhesive and non-adhesive phenotypes by the *in vitro* K99-mediated adhesion to isolated small-intestinal brush-border membranes. These phenotypes were linked to the quantity and distribution of receptor-active sialoglycosphingolipids (D. Seignole, et al. submitted). Nevertheless, K99-resistant phenotype was susceptible to other fimbriae types, in particular to K88 infection.

Non acidic sugar-specific lectins.

Information has become recently available on carbohydrate specificities of the other lectins (Table 3). Gal, GalNAc and GlcNAc residues are involved in the specific binding of K88 (47, 134), where Phe^{150} residue is implicated (62). *E. coli* K88 strains do not adhere to brush borders from all piglets, they adhere both *in vivo* and *in vitro* to cells of K88-susceptible piglets but not

TABLE 2. – Mechanisms of action of heat-labile and heat-stable enterotoxins.

Toxin	Action	Effects
LT-I (A1)	activator of adenylate cyclase in a NAD-dependant ADP ribosylation of the GTP-binding protein	
	increase in the PKC activity	increase of cAMP concentration villus cells: absorption of Na^+ inhibited hence of Cl^- and H_2O crypt cells: increase secretion Nka, loss Cl^- and H_2O
LT-II	activator of adenylate cyclase	increase of intracellular cAMP
	as LT-I	fluid secretion
ST-I	activator of guanylate cyclase	
	activator of Ca^{2+}-calmodulin	increase of intracellular level of cGMP secretion of Na^+ and Cl^-
ST-II	cyclic nucleotide independent pathway	active bicarbonate secretion

Pharmacological alteration of the second messenger systems, such as cAMP, cGMP or calmodulin ameliorates secretory diarrhea. A novel selective inhibitor of calmodulin has been tested for antidiarrheal activity in two models of secretory diarrhea (139).

to those of any K88-resistant pigs (133). Four different adhesive phenotypes have been determined with the three variants K88ab, K88ac and K88ad (12, 113). K88ab adhesins adhere to murine brush border membrane proteins with Mr of 57,000, 67,000 and 91,000 (76). Seignole *et al.* (unpublished results) isolated intestinal glycoproteins from piglet brush borders susceptible to the three K88 variants, only to ab and ac variants or entirely resistant to K88 adhesion. Glycoproteins of 67,000 could be related to *b* and *c* determinant-mediated adhesion while 40,000 glycoproteins to the *d* one (132) (Table 3).

Bacterial lectin F41 binds to glycophorin of M or N antigenic type (15), which contains GalNAc residues (84). In bovine asialomucins, it recognizes terminal Gal residues (96). FY fimbriae is a GlcNAc-specific lectin (51, 138). We analyzed FY binding preference by inhibition of bovine erythrocyte MRHA. Disaccharides GlcNAc (β1-4)Gal or GlcNAc(β1-6)Man were found to be poor inhibitors of MRHA whereas GlcNAc(β1-3)Galβ1 was active (unpublished data). These data suggest that FY adhesins internally bind to the following glycan moieties of bovine glycophorins : Gal(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-3)GalNAc-; Gal(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-3) GalNAc-;SA(α2-3)Gal(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal (β1-3)GalNAc described in (74). Cell receptors of 987P lectin are not yet determined. Nevertheless, age-related resistance to disease seems to be associated to the release of the 987P receptors which might facilitate bacterial clearance (28).

Mannose-resistant fimbriae function primarily in the initiation of infection, while the mannose-specific type 1 fimbriae were also found in various strains, acting as recognition determinants in the binding to phagocytic cells, which could play a beneficial role for the host (135, 136).

Small peptide STs and their receptors.

Enterotoxigenic and receptor-binding activities of small peptide ST-Is have been localized within 13 amino acid C-terminal segments (151). Asparagine at position 11 is required for ST-I activity through the formation of hydrogen bonds with receptors and /or functional mediator of guanylate cyclase (101). Heterogeneities of structure and organization of intestinal receptors of ST-I were observed (159). According to crosslinking of labelled toxin with intestinal membranes, putative rat intestinal cell receptors are single polypeptide chains of 160,000 and 136,000 and additional polypeptides composed of at least two 78,000 and 71,000 subunits (60). Multiple proteins could be members of a highly structured receptor complex with a molecular size of 150,000-200,000 (160). It is noteworthy that early in the course of ST binding, ST spontaneously dissociates from its enterocyte receptor (apparent Ka: 10 nM), suggesting that various means of removing bound ST from its receptor may be successful in terminating ST-induced secretion (22).

Blockage of bacteria-host binding: current applications and promising developments.

Blockage of colonization by an indigenous bacterial strain.

TABLE 3. – Fimbriae and receptors.

Pili	HA properties	Receptor affinity
Pili 1(F1)	GP	Mannosides (135) N-glycoprotein (48)
K88(F4)		Gal-X-glycolipid or peptide (7)
K88ab	Ch, GP, P (13)	Protein receptors 57,000; 64,000; 91,000 in brush borders 57,000 and 64,000 in mucus (76)
K88ac	GP (13)	
K88ad	P, GP (13)	
K99(F5)	H, Sh	GM3Gc (104, 141, 157) SPGGc >GM3Gc>GM3Ac (75) glycophorin (83) sialoglycoproteins bovine mucus (96) mucin glycopeptides (82)
F41	Sh, H, GP, Hu	N-acetylgalactosamine (84) Galactose (96)
987P(F6)	no	
FY	Bov	N-acetylglucosamine (51, 138) glycophorin
F42	GH, GP, H, Hu, Sh	receptors Hela cells, pig brush borders (78)
CS31A	no	

Diverse groups of lectins have been distinguished according to primary specificity for different monosaccharides. Bov, bovine; Ch, chicken; GP, guinea pig, H, horse, Hu, human; P, pig; Sh, sheep erythrocytes. Underlined red blood cells (RBC) species are usually used to define the receptor characteristics and evaluate potential inhibitors of adhesion or antigenic activity. 987P and CS31A fail to hemagglutinate Bov, H, GP,Sh, P, and rabbit RBC.

Indigenous bacteria associated with epithelial surfaces are undoubtedly important in exerting certain influences that help the animal host to resist some microbial diseases. Cheng et al. successfully inoculated the animals orally with a mixture of 36 autochthonous species of bacteria and favorably influenced disease resistance and early weight gain (19). Selected strains of *Lactobacillus* species (called probiotics) were often used as dietary supplements with a protective effect against ETEC (40). Oral inoculation of newborn piglets may result in colonization of the intestinal mucosa (111, 156) with specificity for animal host species.

Control of diarrhea by bacteriophages

Examining the dynamics of phage/bacterium *in vivo* Smith *et al.* (145) used phages to control K99 and 987P ETEC-mediated diarrhea. For field use and to avoid incorporation of undesirable host genes in their genomes, the phages chosen do not form a lysogenic relationship with the bacteria strains. Most calves inoculated with ETEC strain and given phages were protected from diarrhea and death because the degree of destruction of ETEC by the phages was sufficient to prevent its proliferation in the small intestine. Emergence of phage-resistant bacteria could be an objection to potential use of phages in therapy, but it seems that the resistant mutants that emerged in the phage-treated animals were markedly less virulent than their parent strains.

In vivo limitation of bacterial virulence factors.

Adaptation of *E. coli* strains to environmental changes is characterized by a shift from a swimming non-fimbriated state to an adhesive fimbriated state (phase variation). The fimbriae phase variation has been identified as an important factor to overcome the host defenses (37, 53, 105, 108). Its on-and-off genetic control is regulated at the transcriptional level; it could be envisaged that further study will lead in the next years to the development of prophylaxes.

Growth temperature, composition of the culture medium, pH and specific growth rate of the bacteria are among the conditions which control fimbriae production (167, 168, 169). A good correlation was observed *in vivo* between intestinal fluid pH and K99 pilus expression, with a concomitant increase of the pH and of the number of piliated bacteria increased from the proximal to the distal portion of the small intestine.

TABLE 4. – Enterotoxins and receptors*

Toxin variants	Carbohydrate-toxin binding strong	less strong	weak	
CT	GM1	GD1b, fucose-GM1 GalGalNAc-GM1b		(45,116)
LTh-I†	GM1	GD1b	GM2, asialo GM1	(45)
LTp-I	GM1			(150)
LT-IIa	GD1b	GM1, GD2	GM2, GM3	(45)
LT-IIb	GD1a, GT1b		GM3	(45)
STp-I	glycoproteins			(60,159,160)

* Sialic acids of gangliosides are *N*-acetylated forms.

† LTh-I variant was isolated from humans.

In contrast, production of K88 is unaffected by low pH (41). Complex and enriched medium tends to suppress the expression of fimbrial adhesins. Acidic dietary supplementation may limit the pilus production and thus limit ETEC colonization as well.

On the other hand, it was observed that some antibiotics in subinhibitory doses alter the bacterial surface hydrophobicity and therefore adhesive capacity of cells. This effect could be due to either a decrease in synthesis or a less efficient expression of the fimbriae (2, 31, 70). Since extensive use of antibiotics has contributed to the development of antibiotic-resistant strains that infect cattle and other animal species, it is not tempting to develop this approach.

Blockage by antibodies.

The fimbriae of ETEC are strongly immunogenic antigens and veterinary experience with vaccines stands as a model. As newborn animals acquire antibodies by ingestion of their dam's colostrum, protection of newborn animals against experimentally induced colibacillosis has been attempted by vaccination of dams and ingestion of colostrum or oral administration of hyperimmune serocolostrum (3, 8, 115, 147). For instance, purified 987P fimbriae were used for vaccination trials (23 pregnant sows which gave birth to 230 piglets) and excellent protection of piglets upon challenge with the homologous 987P positive ETEC was seen (18% diarrhea against 88.6% in control groups) (110). F41 fimbriae has been also shown to be protective (35, 125). K88 and K99 pili were introduced in several commercial vaccines (56). Great heterogeneity of *E. coli* strains means that vaccines must be developed from epidemiological studies on the prevalence of fimbrial antigens occurring among most pathogenic isolates and these vaccines must be able to induce antibodies that block bacterial adhesion (34, 79). In infection of calves by K99, F41, FY positive ETEC, colostrum from cows vaccinated against either K99+F41 or FY fimbriae did not provide protection, but a mixture of the 2 colostra did (24). Use of monoclonal antibodies to pili antigens simplifies the quality control for the incorporation of fimbriae into the multivalent vaccines (161).

On the other hand, production of intestinal antibodies can be stimulated in the young animal, and recent works have concentrated on oral vaccines because oral administration is an efficient method to elicit intestinal secretory antibodies. Since antibody response is often short-lived, its limitations might be overcome by repeated exposure to the antigen administered continually in the feed. Clinical trials confirmed the value of a diet supplemented with *E. coli* antigen.

Protection provided by antibodies to the newborn animal against ETEC-diarrhea is multi-factorial. In response to K88-positive ETEC infection, neutrophil migration into the intestinal lumen and an increased phagocytocis occur in the intestinal lumen of piglets vaccinated with the homologous *E. coli* strain (10). Significant protection obtained in young animals is due to the blocking of adhesive capacity by antibodies. Anti-K99 bovine monoclonal antibody inhibited agglutination of horse erythrocytes (which harbour K99 receptors) in the presence of K99 antigen and inhibited K99 ETEC adhesion to piglet enterocytes (5). The inhibitory effect of hyperimmune serum on the adhesion of *E. coli* K88ab$^+$ is not due solely to antigen-specific reactivity (154). The capacity of immunoglobulins to agglutinate *E. coli* with mannose-specific adhesins suggests that glycan moieties on SIgA molecules act as receptors via lectin-carbohydrate interactions (175).

The fimbriae diversity and risk of emergence of mutant or new classes of adhesins lead to the concept of immunization against the main enterotoxins for simplification of vaccine design. Investigators showed that passive immunity to LT enterotoxin (LT toxoid)

provided a good level of protection for neonatal piglets challenged with LT-producing ETEC strains (43, 46). In order to protect animals against most of the *E. coli* strains producing ST, immunization of pregnant swine with native toxin or synthetic ST-I (which presents the most convenient means for obtaining great quantities), conjugated to a carrier protein were attempted. Protection was obtained, but not as complete as that demonstrated with K99-positive *E. coli* vaccination of dams (42).

Progress in vaccine design.

Advances in genetic and biotechnology provide new approaches to vaccine development based on recombinant DNA techniques and the use of synthetic peptides (21, 56, 158). Possibilities are now emerging for generating bacteria in which specific toxic factors will be deleted or will harbour new combinations of antigens. Moreover, recent knowledge in structure-function relationships permits the design of chimeric proteins and peptides in recombinant pili. These issues include *i)* that immunogenic variable epitopes must be identified to define synthetic peptides corresponding to linear regions of the recombinant in regard to secondary structure and hydrophilicity, *ii)* the identification of regions of fimbriae where variations do not affect the expression of carriers, *iii)* the expression of the foreign gene product, its stability, maximum expression of immunogenicity and absence of toxicity. Therefore, molecular biology techniques established to study the toxins and fimbriae are currently applied to insure high quality, safety and efficacy of genetically engineered vaccines

Alternative approaches to the development of a suitable ST toxoid involve the construction of genetic LT-ST fusion, giving a multivalent toxoid for protection against these two toxins. LT-A::ST-I fusion proteins co-expressed with LT-B in *E. coli* share many properties with native LTs and were recognized by monoclonal antibodies against both components (127). Oral delivery of LT-B/ ST chimeras induces intestinal IgA responses to ST and LT enterotoxins. The B-subunit of LT is not toxic by itself, and the cross-linking technique rendered the ST non toxic (21). Moreover, Clements pointed out that ST antigenicity and immunogenicity were maximum when a seven-amino acid, proline-containing linker sequence (N-P-R-V-P-S-S) was inserted between LT-B and ST.

Investigators are now evaluating the strategy of hybrid fimbriae construction, and results established a promising way in epitope presentation strategy. Synthetic peptide fragments VDAGTVDQTVQLGC derived from type 1 fimbriae, auto-assembled and reached a conformation that gave rise to epitopes which evoked antibodies that reacted with type 1 fimbriae (1). Foreign peptides were cloned in K88ac and K88ad (158). A linear epitope introduced into the pilus subunit was performed, the variable region capable of inducing an immune response was relaced by heterologous antigenic determinants. Different hybrid fimbriae K88-carrying peptides derived from a pilin subunit of *Neisseria gonorrhoeae* and virus were successfully constructed and immunization of mice with hybrid fimbriae raised antibodies specific for the inserted heterologous epitopes (9).

Receptor analogs.

Characterization of the specificity pattern of bacterial lectins is important for designing more effective inhibitors of adhesion to mucosal surface. General methodology may consist of *i)* the screening of receptor specificity and identification of receptor structures, *ii)* the cloning of lectin genes, *iii)* the dissection of detailed binding epitopes and *iv)* extraction or synthesis of receptor analogs.

It was observed by several authors that *in vitro* binding of various pathogenic bacterial surface lectins was competitively inhibited by soluble receptor molecules, glycosides and oligosaccharides (6, 59, 96, 99, 107, 135, 172). It might thus be possible to select substances that can compete with natural receptors for binding. These compounds must be suitable large, non-digestible, nonabsorbable, natural or synthetic receptor substances do not lead to increased adhesion by their incorporation into epithelial cell membrane.

Since most natural foodstuffs contain sugars, polysaccharides, proteins, glycoproteins and glycolipids, many of these substances should antagonize the adhesion of lectins of *E. coli* and *Streptococcus pneumoniae* or *Haemophilus influenzae* (6, 7, 148, 172). Milk fat was a target for K88 fimbriae (7) and can interfere with the attachment of ETEC adhesins. Bovine skim milk, commercial casein and murine milk share the property of binding ST-I toxin (148). Since blood plasma contains a variety and a great amount of protein-bound oligosaccharides, free oligosaccharides and glycopeptides may well inhibit the binding of adhesins. Soluble receptor analogs issued from cattle blood serum, plasma-derived glycoprotein glycans of the *N*-acetyllactosamine type, bound to ETEC and effectively inhibited *in vitro* attachment (97). Binding to a soluble molecule present in the lumen may prevent binding to the same epitope in a membrane-bound form. Local concentrations must be high enough to compete successfully with the cooperative binding of piliated bacteria (96). When the receptor analogs are administrated to colostrum-deprived newborn calves following challenge with K99-positive ETEC, the level of gut colonization is significantly decreased by a factor of 100. These compounds thereby minimized acuteness of K99-mediated infection (97). Clinical studies of farm calves showed that continuous feeding of receptor analogs during periods of risk of infection protected young animals. A variety of sugar sequences present in plasma permits a combination of series of receptor analogs necessary to inhibit the adhesins exposed together at the bacterial surface.

Chemical modification may produce a molecule with an even higher affinity for pathogens than that of the native receptor. Pseudopolysaccharides, artificial NeuAc-conjugates that bind to agglutinins, antibodies and virus are highly immunogenic in rabbits (122). The capacity of artificial glycoconjugates to serve as inhibitors of hemagglutinin properties are the subject of actual research.

Selection

Host inheritable determinants of membrane glycoproteins and glycolipids define the avidity of ETEC for the intestinal cells. Biochemical characterization of relevant receptors revealed qualitative and quantitative differences in receptor expression (157). As mentioned above, phenotypic-related susceptibility has been demonstrated in piglets (12, 113, 146). Differences in susceptibility between races have been observed, thus Chinese piglets mainly belong to a non-adhesive phenotype (18). It may be possible to breed disease-resistant live-stock, however, the recessive gene of resistance to K88 ETEC infections is variably detected. Prevention could be obtained by the selection of genetically resistant individuals. Nevertheless, in an endemic situation, genetically susceptible sows protect their susceptible offspring from natural infection. In the same time, susceptible piglets born to resistant dams are at a greater risk of infection, since K88-resistant dams are never colonized by K88 ETEC. Actually, one way of reducing the level of neonatal diarrhea in a pig population is to bred homozygous resistant boars with heterozygous sows. Protection is genetically or from the dam's colostrum (133).

Concluding remarks to some remaining questions

"Is molecular epidemiology a germ theory for the end of the twentieth century?" ask Loomis and Wing (86). For some, disease can be prevented by altering the environment without the need of detailed knowledge of pathogenic mechanisms while other researchers argue for molecular biology and mechanistic epidemiology. The authors propose that the integrative theory will be "the challenge for epidemiologists facing the 21st century. This entails embracing both new advances from laboratory science and old concepts about the role of environment without isolating either one from the whole systems of which they are an essential part.".

Three study levels in host cell-bacteria interactions are currently developed, the molecular, cellular and physiologic levels (77). Multidisciplinary approaches involving biologists, chemists, molecular biologists, geneticists and epidemiologists are needed to identify the interacting molecules. The complex situation is only scarcely understood and warrants further investigation that may open new possibilities to modulate or control the adhesion of pathogenic bacteria in a way that will lead to prolonged and increased therapeutic actions. From work that has as yet practical applications in therapy have emerged a number of relevant points in diarrhea vaccine design. Knowledge of the sequences of the receptors will aid in the design of receptor analog inhibitors of adhesion. N. Sharon prophesies "the day may come when inhibitors of lectin-mediated adherence will be used clinically to prevent colonization before the bacteria have had the chance to overwhelm the host" (135).

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