Original article

Putative roles of the CNF2 and CDTIII toxins in experimental infections with necrotoxigenic *Escherichia coli* type 2 (NTEC2) strains in calves

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Abstract

Newborn colostrum-restricted calves were orally inoculated with an *Escherichia coli* strain, identified originally as non-pathogenic, and into which the plasmid pVir was conjugally transferred. This resulted in diarrhea, intestinal lesions and extra-intestinal invasion, suggesting that factors affecting these pathogenic properties are located on pVir. In order to analyze the respective roles of the toxins CNF2 and CDTIII in the pathogenesis, colostrum-restricted calves were inoculated with isogenic mutants in the *cnf2* and the *cdt-III* genes. The loss of *cnf2* is associated with a reduction in the pathogenicity, since diarrhea does not occur in calves challenged, in spite of successful colonization of the intestine. Nevertheless, the mutant strain remains able to invade the bloodstream and to localize in the internal organs. Conversely, the calves inoculated with mutant in the *cdt-III* gene evolved in the same way as wild-type strain-inoculated calves with regard to clinical signs and macroscopic or microscopic lesions.

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1. Introduction

Necrotoxigenic *Escherichia coli* (NTEC) is characterized by the production of a toxin called cytotoxic necrotizing factor (CNF) [1,2]. This name corresponds to its capacity to induce multinucleation in cell culture and to cause necrosis in rabbit skin [3]. Two different types of NTEC have actually been reported on the basis of the production of CNF1 and CNF2 toxins: NTEC1 and NTEC2 [3,4].

The *cnf1* gene is located on the chromosome [5], and the *cnf2* gene, on a plasmid, which was identified as the Vir plasmid [6,7]. The Vir plasmid also carries genes encoding other putative virulence factors: the F17b and the F17c subtypes of the F17 fimbrial adhesin family [8], a new member of the Afa afimbrial adhesin family, Afa-VIII [9], and/or a new member of the cytolethal distending toxin (CDT) family: CDT-III [10].

Although the effect of the CNF toxins consists of the formation of multinucleated cells, the effect of the interaction between NTEC2 strains and HeLa cells is characterized by the production of giant mononucleated cells with the appearance of actin stress fibers and a block in the G2/M phase of cell division [10]. The actin cytoskeleton reorganization is mediated by the CNF2 toxin [11], but the cell cycle arrest in G2/M phase is caused by CDT-III [10,12].

In 1974, Smith showed that both wild-type CNF-positive and a non-pathogenic strain carrying the Vir plasmid after conjugation (H209(pVir)) caused death by cardiac failure after intravenous inoculation in chicken [7]. In rabbits, inoculation of intestinal ligated loops with both the wild-type CNF1-producing strain and C600 (CNF1+) caused frank mucoid diarrhea and pathologic intestinal findings in the small and large intestine, including necrosis, hyperplasia and inflammation. However, the use of a CNF-defective mutant did not significantly affect the onset, duration or severity of the diarrhea, although associated with a decrease in intestinal inflammation [13].

In the same way, newborn colostrum-deprived germ-free piglets orally inoculated with a wild-type CNF1-producing strain showed pulmonary lesions (consolidation and inflammation) and colonization of intestinal and extra-intestinal

organs (mesenteric lymph nodes, lungs, liver, spleen and kidneys). But, the inactivation of *cnf1* induced only a weaker colonization of the ileum and a slight decrease in inflammatory response in the lungs [14].

In 2001, we orally challenged newborn colostrum-restricted calves with two NTEC2 strains: 1404 (CNF2, CDT-III, F17b) and B20a (CNF2, CDT-III, F17cd). Both strains were able to colonize the gut and to cause enterocolitis expressed by watery diarrhea. In addition, they were able to invade the bloodstream and to localize in internal organs (mesenteric lymph nodes, liver, spleen and lungs), causing lymphadenitis and bronchopneumonia [15]. However, the influence of the different virulence factors in the pathogenesis of those NTEC2 strains was not investigated.

In the present paper, we study the role of the Vir plasmid, and subsequently, of the toxins CNF2 and CDT-III in our calf model of infection [15] by comparing the clinical signs and lesions caused by a transconjugant of a non-pathogenic strain (H209) carrying the Vir plasmid of strain 1404 and NTEC2 strain 1404 mutants in the *cnf2* and *cdt-III* genes.

2. Materials and methods

2.1. Bacterial strains

NTEC2 strain 1404 was isolated from the blood of a calf with septicemia [16]. This strain belongs to serotype O78:K80, produces an aerobactin [16] and harbors genes encoding the CNF2 toxin, CDT-III and the F17b fimbriae [8,15,16].

Non-polar insertional mutations of *cnf2* or *cdt-III* were produced by allelic exchange in *E. coli* strain 1404. A chloramphenicol (cm) or a kanamycin (kn) resistance cassette was inserted into *cnf2* or *cdt-IIIA* using standard procedures [17], as described by Fournout et al. [14] for the *cnf1* gene (Oswald, unpublished). The resulting mutant strains were 1404 *cnf2::cm* (1404ΔCNF2) and 1404 *cdt-IIIA::kn*

(1404ΔCDT-III), respectively. They were confirmed by polymerase chain reaction and Southern-blot analysis (Oswald, unpublished).

Strain H209 was isolated from the feces of a healthy human [7]. This strain is serotype O9:K31:H14 and is considered non-pathogenic [7,10].

Strain H209 (pVir) is a transconjugative strain. The virulence plasmid of strain 1404 was mobilized in strain H209 with the use of the conjugative plasmid pF'Cm^R [18], according to the protocol described by Smith in 1974 ([7,10] Oswald, unpublished).

2.2. Calf infections

Eleven naturally born colostrum-restricted calves were inoculated at 6 h of age with 10^9 organisms of the appropriate challenge strain: four received H209 (pVir), four received 1404ΔCNF2 and three received 1404ΔCDT-III (Table 1). The experimental protocol was performed as previously described [15]. One control calf (C) received 250 ml of sterile saline without bacteria and was kept in the same conditions as the inoculated calves.

2.3. Necropsy

The calves were euthanized between 44 and 91 h postinoculation (pi) (Table 1) by parenteral injection of sodiumpentobarbital 20% and were necropsied immediately. The necropsy procedure and the histopathological examination were performed as previously described [15].

2.4. Bacteriology

The presence of coliforms in feces and intestinal contents was quantitatively estimated after inoculation of Gassneragar plates (Merck, Darmstadt, Germany) with a spiral plater (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) and overnight incubation at 37 °C. In addition, the proportion

Table 1
Correlation between the presence of diarrhea and the challenge strains in the feces

Calves	Challenge strain	Euthanasia (pi)	Diarrhea		Challenge strain in the feces	
			Appearance (pi)	Persistence until	Appearance (pi)	Persistence until
T1	H209 (pVir)	44	20	e	20	e
T2	H209 (pVir)	48	32	e	32	e
T3	H209 (pVir)	50	24	e	24	e
T4	H209 (pVir)	60	28	e	28	e
M1	1404ΔCNF	91	–	–	16	e
M2	1404ΔCNF	52	–	–	16	e
M3[a]	1404ΔCNF	54	36	e	16	e
M4	1404ΔCNF	62	–	–	20	e
M5	1404ΔCDT	52	24	e	16	e
M6	1404ΔCDT	46	28	e	16	e
M7	1404ΔCDT	48	24	e	12	e
C	–	–	–	–	nr	nr

pi, hours post-inoculation; e, euthanasia; –, none or negative; nr, not relevant.
[a] Rotavirus was present in the feces.

of the challenge strains among lactose-fermenting colonies was quantitatively estimated by colony hybridization of a plate with radioactive probes for CNF2 [19] or CDT-III ([20], Pérès and Oswald, unpublished), as previously described [15]. The presence of coliforms in extra-intestinal tissues and heart blood was estimated qualitatively: a tissue sample or a drop was directly plated onto Gassner-agar and Colombia with 5% sheep blood–agar plates (Becton–Dickinson, Meylan, France). The plates were incubated overnight at 37° C, and colonies were identified by colony hybridization with radioactive probes for CNF2 [19] or CDT-III ([20], Pérès and Oswald, unpublished), as previously described [15]. Diarrheic fecal samples were also examined for the presence of coronavirus, rotavirus, *Cryptosporidium parvum*, K99[+] *E. coli* and salmonellae, as previously described [15].

3. Results

3.1. Clinical signs

The four calves infected with H209 (pVir) (T1, T2, T3, and T4), the three calves inoculated with 1404ΔCDT-III (M5, M6, and M7) and one calf inoculated with 1404ΔCNF2 (M3) developed watery diarrhea, which appeared between 20 and 32 h post-inoculation and persisted until euthanasia (Table 1). One calf (T1) also had general clinical signs including apathy and anorexia. In contrast, three of the four calves inoculated with 1404△CNF2 (M1, M2, and M4) and the control calf (C) did not suffer from any diarrhea and remained clinically healthy (Table 1).

3.2. Excretion of the challenge strain

The challenge strains (1404ΔCNF2, 1404ΔCDT-III or H209 (pVir)) were recovered from fecal cultures of all inoculated calves (Table 1). In diarrheic calves, the fecal excretion coincided with (in the H209 (pVir)-inoculated calves), or was observed before (in the 1404ΔCNF2- and 1404ΔCDT-III-inoculated calves), the appearance of the diarrhea (Table 1). In all calves, the excretion persisted until euthanasia (Table 1). The proportions of challenge strains among *E. coli* were highly variable and were not correlated with the presence of clinical signs (data not shown). All diarrheic calves were negative for the presence of cryptosporidiae, K99[+] *E. coli*, salmonellae or coronavirus. Only calf M3 was positive for the presence of rotavirus.

3.3. Necropsy and microscopic lesions

At necropsy, the calves had lesions of vascular congestion and thickening of the intestinal mucosa in the jejunum and the ileum, and hypertrophy of the associated mesenteric lymph nodes (MLNs). Light microscopic examination of the small intestine showed congestion and atrophy of the villi in all calves, associated with hemorrhages in calves T2, T3 and T4. High infiltration of inflammatory cells (neutrophils, lym-

phocytes and plasmocytes) was present in the mucosa. It also showed abscessation or necrosis of the crypts, and lymphatic canals were distended. Peyer's patches showed activation in the ileum (all calves) and sometimes in the jejunum (M6 and M7).

The caecum and the colon showed only congestion and some lymphocytes in the mucosa of all calves. Nevertheless, in calf M3, an infiltration of inflammatory cells (lymphocytes and neutrophils) was present in the mucosa and the submucosa.

No macroscopic lesions were observed in other organs. Light microscopic examination of the lungs showed infiltration of inflammatory cells (lymphocytes and/or neutrophils) along with interalveolar edema in the interstitium. The bronchial tubes remained intact.

The mesenteric lymph nodes of all calves showed congestion and activation of lymphoid nodules. They contained neutrophils associated with lymphocytes in some calves (M4, T3 and T4). The presence of inflammatory cells was also observed in the liver of the H209 (pVir)-inoculated calves. This infiltration was associated with hemorrhages in three of them (T2, T3, T4).

3.4. Bacteriology at necropsy

Bacteriological examination of the intestinal content showed the presence of the challenge strains (1404ΔCNF2, 1404ΔCDT-III or H209 (pVir)) in high numbers from various parts of the intestine in all calves (Table 2), proportions among *E. coli* were highly variable (data not shown). They were also recovered in pure culture from the heart blood, the lungs and/or the liver of the 1404ΔCNF2-, 1404ΔCDT-III- and the H209 (pVir)-inoculated calves and from the spleen and/or the MLNs of the calves inoculated with 1404ΔCNF2 or 1404ΔCDT-III (Table 2).

Table 2
Recovery of the challenge strain from the intestinal content and extra-intestinal organs at necropsy

Number of calves	4	4	3	3
Challenge strain	H209 (pVir)	1404 ΔCNF2	1404 ΔCDT-III	1404 wild-type [15]
Duodenum[a]	6.6	8.2	7.3	7.0
Jejunum[a]	8.6	9.0	8.1	8.4
Ileum[a]	8.7	8.8	8.5	8.6
Caecum[a]	9.0	8.9	8.6	8.4
Colon[a]	8.2	8.9	8.4	8.2
Blood[b]	2/4	1/4	0/3	2/3
Lung[b]	3/4	2/4	3/3	2/3
Liver[b]	3/4	2/4	2/3	3/3
Spleen[b]	0/4	1/4	0/3	0/3
MLN[b]	0/4	3/4	2/3	0/3

[a] Mean value of bacterial counts in log colony-forming units ml^{-1} of intestinal content.

[b] Number of positive calves/number of challenged calves.

4. Discussion

Inoculation of four colostrum-restricted newborn calves with the H209 (pVir) strain results in successful colonization of the intestines, mainly the jejunum and the ileum and in occurrence of diarrhea correlated with the excretion of the challenge strain in the feces. Those calves also presented signs of invasion, as the challenge strain was isolated from the heart blood and from internal organs. Histological lesions were present in the intestines. They were characteristic of enteritis, including congestion, infiltration of inflammatory cells and abscessation of the crypts. The four calves inoculated with strain H209 (pVir) thus evolved in the same way as 1404 wild-type-inoculated calves challenged in a preliminary study [15]. This confirms the hypothesis that factors conferring the ability to cause diarrhea and intestinal lesions in calves as well as the ability to invade organs are located on the Vir plasmid. Related observations were made by Smith [7], since H209 (pVir) caused death by cardiac failure in chicken, whereas H209 did not, and is in any case, considered non-pathogenic [10].

In order to analyze the respective roles of the toxins CNF2 and CDT-III in the pathogenesis, colostrum-restricted calves were inoculated with isogenic mutants in the *cnf2* and the *cdt-III* genes. The loss of *cnf2* is associated with a reduction in the pathogenicity, since diarrhea did not occur in three calves challenged in spite of the successful colonization of the intestine. In those calves, the fecal excretion of the challenge strain started even earlier than in wild-type- and transconjugant-challenged calves and lasted until the time of euthanasia. In the fourth calf, diarrhea was observed, but it was probably due to another factor, since a rotavirus was recovered from the diarrheic feces of this calf.

Surprisingly, in all calves, the enteritis lesions were still present. However, they could be due to other structures of host bacteria, such as other "virulence properties" or surface structures. Indeed, a calf inoculated under the same conditions with the strain 25KH09 (O101:K+, pVir, F17a+) showed slight inflammation with congestion, atrophy of the villi, and lymphocyte infiltration of the small intestine (15). In contrast, no abnormalities other than congestion were found in the intestine of a calf inoculated with strain C600 (O–, K–) (unpublished data). In addition, congestion is a frequent artefact produced by pentobarbital sodium euthanasia [21]. The mutant strain was still able to invade the bloodstream and to localize in the internal organs, including the spleen and MLNs.

Conversely, all three calves inoculated with 1404ΔCDT-III evolved in the same way as wild-type strain-inoculated calves with regard to clinical signs, macroscopic or microscopic lesions. This can be explained in different ways. CDT-III could play a role not in the pathogenicity itself, but only in the potentialization of the effects of CNF2 by facilitating invasion, for example. Or, CDT-III could play a role in the colonization process, and this effect is masked by the presence of the F17 adhesin. Or, the mutation in the CdtA

subunit is not efficient in this case, as CdtB by itself is capable of recapitulating all the toxic effects of the CDT holotoxin when microinjected into host cells, whereas CdtA is more implicated in the translocation of the toxin [22].

In conclusion, our results strongly suggest a role of the CNF2 toxin in the occurrence of diarrhea in newborn calves, in contrast to CNF1 [13,14] and CDT-III, but neither in the development of bacteremia nor apparently in the survival of the NTEC2 strains in the bloodstream or internal organs.

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