Helicobacter pullorum Cytolethal Distending Toxin Targets Vinculin and Cortactin and Triggers Formation of Lamellipodia in Intestinal Epithelial Cells

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Helicobacter pullorum, a bacterium initially isolated from poultry, has been associated with human digestive disorders. However, the factor responsible for its cytopathogenic effects on epithelial cells has not been formally identified. The cytopathogenic alterations induced by several human and avian H. pullorum strains were investigated on human intestinal epithelial cell lines. Moreover, the effects of the cytolethal distending toxin (CDT) were evaluated first by using a wild-type strain and its corresponding cdtB isogenic mutant and second by delivering the active CdtB subunit of the CDT directly into the cells. All of the H. pullorum strains induced cellular distending phenotype, actin cytoskeleton remodeling, and G2/M cell cycle arrest. These effects were dependent on the CDT, as they were (1) not observed in response to a cdtB isogenic mutant strain and (2) present in cells expressing CdtB. CdtB also induced an atypical delocalization of vinculin from focal adhesions to the perinuclear region, formation of cortical actin-rich large lamellipodia with an upregulation of cortactin, and decreased cellular adherence. In conclusion, the CDT of H. pullorum is responsible for major cytopathogenic effects in vitro, confirming its role as a main virulence factor of this emerging human pathogen.

Keywords. isogenic mutant; epithelial cells; lentivirus; lamellipodia; actin; Helicobacter pullorum; cytolethal distending toxin; cortactin; vinculin; adherence.

Helicobacter pullorum is a bile-resistant Helicobacter species that was isolated initially from the liver, duodenum, and cecum of asymptomatic poultry [1]. This enterohpatic Helicobacter species has also been isolated from patients with gastroenteritis [1–4] and may be associated with inflammatory bowel disease [5–7], as well as with chronic liver disease [8–13].

As for other enterohpatic Helicobacter species, very few data are currently available on the pathogenesis of H. pullorum, mainly because of difficulties in cultivating this fastidious organism. In addition, no transformation of H. pullorum has yet been reported, and no mutant has been described. However, in vitro coculture experiments on gastric and intestinal epithelial cells show upregulation of the proinflammatory cytokine interleukin 8 by H. pullorum, requiring bacterial adherence and involving the nuclear factor-кB pathway, probably via its lipopolysaccharide, as shown by a high Limulus test reactivity [14, 15]. Recent results suggest that H. pullorum possesses a type VI secretion system that may interact with endocytic vesicles and may trigger adherence to intestinal epithelial cells [16]. In addition, in vitro and in vivo data indicate that some enterohpatic Helicobacter species, such as Helicobacter hepaticus, Helicobacter bilis, Helicobacter canis, Helicobacter...
marmotae, Helicobacter winghamensis, Helicobacter mastomysri-
nus, Helicobacter cinaedi, and H. pullorum, secrete a common
bacterial virulence factor, cytolethal distending toxin (CDT)
[17–22]. Studies in mouse models revealed that the CDT of H.
cinaedi and H. hepaticus promotes colitis in interleukin 10–
deficient mice [23, 24] and that the CDT of H. hepaticus
induces liver inflammation via its CdtB subunit and promotes
the development of dysplasia in A/JCr mice [25].

Currently, 2 studies on the pathogenic role of H. pullorum
CDT are available, both of which were performed with crude
bacterial sonicates [21, 26]. In fact, research concerning the
pathogenicity of CDT encounters difficulties in the production,
purification, and penetration of the toxin into the target cell.
Indeed, it is necessary to produce and purify sufficient amounts
of the CdtA, CdtB, and CdtC subunits to reconstitute a com-
plete active toxin. Unfortunately, an adequate quantity of the
active CdtB subunit is difficult to produce as a recombinant
protein because of its toxicity in numerous recombinant ex-
pression systems. Even the cell-free protein expression system
for CdtB, which we previously described [27], did not yield ac-
ceptable quantities, because the DNase activity of the neo-
synthesized CdtB hydrolyzed the vector used for its production.
Moreover, H. pullorum culture makes it hard to achieve large-
scale production of this slow-growing bacterium. These prob-
lems constitute a technological barrier that limits the work on
this toxin.

The present study aimed to evaluate the cytopathogenic effects of the CDT of H. pullorum by developing a 2-way origi-
nal system composed of (1) a cdtB isogenic mutant H. pullorum
strain and (2) a lentivirus-based system for expressing CdtB
subunit directly into the epithelial cells. This latter system at-
tributed the observed effects specifically to the toxin. In both
systems, cellular proliferation, the cell cycle, remodeling of the
actin cytoskeleton, the microtubule network, and focal adhe-
sions were evaluated.

MATERIALS AND METHODS

Cell lines and H. pullorum strains, reagents and antibodies, the
sequencing of the cdtB locus of H. pullorum, the construction of the H. pullorum cdtB isogenic mutant, the construction of the
plasmid used for the lentivirus production, lentivirus produ-
don, May-Grünwald Giemsa and immunofluorescence
staining, cell proliferation, G2/M transition and adherence
assays, Western blotting, primer designs, real-time polymerase
chain reaction (PCR), and statistical analyses are described in
the Supplementary Materials.

Coculture and Transduction Experiments

Caco-2, HCA-7, and HT-29 cells were seeded on culture plates
or glass coverslips 24 hours before addition of bacteria or lenti-
virus preparations to the culture medium at a density defined
for each experiment (see the Supplementary Materials). For co-
culture experiments, the culture medium was removed, a
volume corresponding to a multiplicity of infection (MOI) of
100 bacteria/cell in renewed medium with fetal calf serum
(FCS) was added, and incubation was continued for 72 hours.
For some coculture experiments, bacteria were seeded on semi-
permeable tissue culture inserts (pore diameter, 0.2 µm;
Anopore, Nunc, Naperville, IL) fitted into culture wells con-
taining epithelial cells. For lentivirus-based transductions, the
culture medium was removed, and volumes corresponding to a
MOI of 30 viruses/cell in FCS-free renewed medium were
added to each cell culture well for 12 hours. Then, FCS was
added to a final concentration of 10% for another 12 hours,
medium was completely renewed with fresh medium with FCS,
and incubation was continued for 48 hours, corresponding to a
final transduction incubation time of 72 hours.

RESULTS

Effects of H. pullorum on Cell Morphology

The effect of 10 H. pullorum strains of human (n = 5) and avian
(n = 5) origin was evaluated in coculture experiments (72
hours) with the human intestinal epithelial cell lines HT-29,
Caco-2, and HCA-7. All H. pullorum strains induced similar
changes in the morphology of the Caco-2 and HCA-7 cells,
which appeared enlarged with distended or multiple nuclei
after 72 hours of coculture (Figure 1A and 1B). The cell cycle
analysis revealed an increase in the percentage of cells in G2/M
phase only in H. pullorum–infected cells (human NCTC 13157
and avian CCUG 33840 strains; Figure 1C), the increase being
more important for the human strain NCTC 13157. The per-
centage of cells blocked by the CDT during the G2/M phase
was low but corresponds to that previously reported [26]. None
of these effects was observed in HT-29 cells (data not shown).
The distending phenotype was also observed after treatment
with a filtered bacterial supernatant or after coculture in a
Transwell system preventing contact between bacteria and
cells but allowing the diffusion of soluble factors (Figure 1D).
These results suggest that the bacterial factor responsible for
the distending phenotype is a soluble factor secreted by H. pul-
lorum.

Two soluble factors involved in cytotoxic activity were re-
ported in H. pullorum: the CDT toxin [21, 26] and a soluble
toxic factor, still unidentified, causing a mitotic catastrophe
resulting in primary necrosis of hepatic cells [28]. Thus, the pre-

cence of the cdtB gene (825 bp) in these 10 H. pullorum strains
was confirmed by sequencing (GenBank accession numbers
available in Supplementary Materials), suggesting that all of
these strains encode a putative CdtB protein of 274 residues. As
expected, the CdtB protein of H. pullorum was the most closely
related to those of other Helicobacter species (H. winghamensis,
H. bilis, H. hepaticus, and H. mastomysriximus), with 69%–75%
identity, whereas those of H. cinaedi (51% identity) and Campylobacter species (52%–57% identity) showed less sequence identity. When comparing the sequence of the cdtB gene among the H. pullorum strains, including the MIT98–5489 strain, some nucleotide polymorphisms were observed. However, these polymorphisms did not cluster according to the geographical or host origin of the strains (Supplementary Figure 1A). In addition, most of the polymorphisms observed at the gene level correspond to “synonymous” substitution rates. In conclusion, the CdtB is highly conserved at the protein level among H. pullorum strains (Supplementary Figure 1B and 1C), with identity ranging from 99% to 100% (over 274 residues), explaining the similar percentages of cells with distended nuclei obtained in response to these strains (Figure 1B).

Figure 1. Effects of Helicobacter pullorum on human intestinal epithelial cells. All the data were obtained after a 72-hour coculture with H. pullorum. A, Morphological changes induced by H. pullorum strains NCTC 13157 (human origin) and CCUG 33840 (avian origin) on Caco-2 and HCA-7 cells revealed by May Grünwald-Giemsa staining. Arrows indicate enlarged Caco-2 and HCA-7 cells with distended nuclei or multinucleated. Scale bar, 50 µm. B, Quantification of Caco-2 and HCA-7 cells displaying a distended phenotype without (control, white) or with H. pullorum strains of human (n = 5, black) and avian (n = 5, grey) origin. Data represent the percentage of cells with distended nuclei determined after labeling with the Hoechst 33342 compound, as well as the mean results obtained with human and avian strains. C, Analysis of DNA contents of Caco-2 and HCA-7 cells incubated without (control) or with H. pullorum strains NCTC 13157 (human origin) and CCUG 33840 (avian origin). Cells were fixed and labeled with DAPI (4′,6′-diamidino-2-phenylindol) and analyzed by flow cytometry. D, The same experiment as in panel B was performed with H. pullorum strains NCTC 13157 (human origin) and CCUG 33840 (avian origin) in the presence or absence of Transwell inserts or with filtered bacterial culture supernatants of the same strains. Strain information and GenBank accession numbers of the cdtB sequences are presented in the Supplementary Materials. A and C, Data represent 1 representative experiment of 3. B and D, Data represent the mean of triplicates in 1 representative experiment out of 2. The discontinuous line shows the basal percentage of cells with distended or multiple nuclei under control conditions. *P < .05 versus control.
Figure 2. Effects of inactivation of the cdtB gene on Helicobacter pullorum–induced cytodistending phenotype and on cell proliferation. A, Analysis of H. pullorum–transformed strain CCUG 33840 (avian origin) by polymerase chain reaction (PCR) and Southern blot hybridization (Supplementary Materials). The correct insertion of the kanamycin cassette at the expected locus of the H. pullorum genome was first confirmed by PCR with external primers, with subsequent analysis of the PCR products on a 1% agarose gel stained with ethidium bromide. Then, the unique insertion of the kanamycin cassette into the H. pullorum genome was verified by Southern blotting. SspI-digested genomic DNA was separated on a 0.8% agarose gel, and Southern blotting was performed with a 172-bp labeled probe designed from the kanamycin gene aphA-3. A 1-kb DNA ladder (Invitrogen) was used. B, The percentage of cells displaying a distended phenotype was determined by fluorescence microscopy after labeling with the Hoechst 33342 compound after 72-hour coculture of Caco-2 and HCA-7 cells without (control, white) or with (grey) H. pullorum wild-type strain CCUG 33840 (avian origin) and the corresponding isogenic cdtB mutant. Data represent the percentage of Caco-2 and HCA-7 cells with distended nuclei (enlarged or multinucleated). C, Cell proliferation of Caco-2 and HCA-7 cells was determined after 48 hours (for HCA-7) or 72 hours (for Caco-2) of coculture without (control, white) or with (grey) H. pullorum wild-type.
To attribute the observed cytopathogenic effects to CdtB and to evaluate a possible cytopathogenic effect induced by the un-identified factor reported in *H. pullorum* [28], 2 types of experiments were performed. First, a *cdtB* knockout (KO) strain was constructed, and the effects induced by this KO strain were compared to those induced by the wild-type (WT) strain. Second, a lentivirus vector was produced to deliver the CdtB subunit of *H. pullorum* directly into host cells.

**Construction of a cdtB Isogenic Mutant of *H. pullorum***

A construction containing the *cdtB* gene truncated by a kanamycin resistance cassette was integrated into the *H. pullorum* chromosome by double homologous recombination in natural transformation experiments (Supplementary Materials). Among the 10 *H. pullorum* strains tested, only the avian strain CCUG 33840 was recovered after transformation. An isogenic mutant for the *cdtB* gene was obtained for this strain with both a plasmid and PCR products. Approximately 500 bp in the flanking regions on each side of the kanamycin cassette (1243 bp) were sufficient to allow the insertion of the cassette in the *H. pullorum* genome. PCR and sequencing of the CDT locus indicated that the insertion was accurate, with no substitution, insertion, deletion, nor mutation (Figure 2A; sequence not shown). In addition, Southern blot hybridization confirmed that no illegitimate insertion of the kanamycin cassette occurred elsewhere in the *H. pullorum* genome, since the expected unique 1610-bp band was observed (Figure 2A).

**Consequence of Inactivation of the cdtB Gene on *H. pullorum*–Induced Cytopathogenic Effects**

Coculture experiments revealed that the increased nuclear dis- tension induced by *H. pullorum* CCUG 33840 WT strain on Caco-2 and HCA-7 cells was not observed with the corresponding *cdtB* KO strain (Figure 2B). Similarly, the inhibition of cellular proliferation and the increase of cells in G2/M phase induced by *H. pullorum* were not observed with the corre- sponding *cdtB* KO strain (Figure 2C and 2D). In conclusion, none of the effects reported for the WT strain were observed with the *cdtB* KO strain, suggesting that CdtB induces these cytopathogenic effects.

**Effects of CdtB Expressed Via a Lentivirus-Based Strategy on Cellular Morphology**

The *cdtB* gene of *H. pullorum* strain CCUG 33840 fused at its 3′ end to 3 repeats of the influenza virus hemagglutinin (HA) epitope (CdtB-3HA) was cloned into pLVTHM instead of the enhanced green fluorescent protein (GFP) gene initially present in this vector. Both plasmids allowed the production of lentivirus particles PV-CdtB and PV-GFP that were used to transduce Caco-2 and HCA-7 cells at a MOI of 30. Transduction efficiency was analyzed by flow cytometry (GFP fluorescence), Western blot, and fluorescence microscopy (HA and GFP immunodetection). The results of flow cytometry obtained on Caco-2 and HCA-7 cells revealed high percentages of GFP-positive cells after 48 hours (94% ± 3% for both cell lines) and 72 hours (>96% for both cell lines) of transduction with PV-GFP. Both GFP and CdtB expression was detected at the expected Mr by Western blotting in the respective transduced cells (Figure 3A). As expected, immunofluorescence experiments revealed that CdtB was detected mainly in the nucleus and especially around the nucleolus (Figure 3B). Interestingly, CdtB was also detected at the cell periphery lamellipodia and membrane ruffles (Figure 3B). Microscopy analysis of Caco-2 and HCA-7 cells (Figure 4A) revealed distending phenotypes similar to those observed with *H. pullorum* in coculture experiments (Figures 1A, 1B, and 2B) but at higher percentages (Figure 4B). Similarly, a significant inhibition of the prolifera- tion (Figure 4C) and increase in the percentage of cells in G2/M phase (Figure 4D) were observed in response to CdtB in both cell lines, also at higher percentages as compared to those observed with *H. pullorum* in coculture experiments (Figure 2C and 2D). Together, these results suggest that lentivirus expres- sion of CdtB in epithelial cells induces effects similar to those observed with *H. pullorum*, thus confirming that the effects ob- served in coculture experiments are due to CdtB.

Interestingly, similar effects were observed in PV-CdtB–transduced HT-29 cells (Supplementary Figure 2), whereas this cell line was not susceptible to CdtB when using *H. pullorum* strains in coculture experiments.

**Effect of CdtB of *H. pullorum* on the Actin Cytoskeleton**

The effects of CdtB on the actin cytoskeleton were explored on Caco-2 cells by immunofluorescence microscopy. Infection with *H. pullorum* WT strain (Figure 5A) or transduction with PV-CdtB (Figure 5B) induced a cellular and nuclear enlarge- ment, with the formation of cortical actin-rich large lamellipo- dia and an increased staining intensity of vinculin that appeared delocalized from focal adhesions to the perinuclear region in the cytosol. These effects were not observed in the re- spective controls and in cells infected with the *cdtB* KO strain or transduced with the PV-GFP. α-tubulin staining revealed a moderate polarization disturbance of the microtubule network inside the cellular body (Figure 5A and 5B). Similar results were obtained in response to 2 other strains of human origin (CCUG 33839 and NCTC 13157; Supplementary Figure 3A).

Figure 2 continued. (WT) strain CCUG 33840 (avian origin) and the corresponding isogenic mutant. A, Analysis of DNA contents of Caco-2 and HCA-7 cells after 72-hour coculture without or with *H. pullorum* strain CCUG 33840 (avian origin) and the corresponding *cdtB* isogenic *H. pullorum* strain (ΔcdtB). Cells were fixed and labeled with DAPI (4′,6′-diamidino-2-phenylindol) and analyzed by flow cytometry. Data represent 1 representative experiment of 3. B and C, Data represent the mean of triplicates in 1 representative experiment of 3. The discontinuous line shows the basal rate in control cells. *P* < .05 vs control.
and also in HCA-7 cells (Supplementary Figure 3B). Moreover, cortactin, a component of the cortical actin-rich lamellipodia and membrane ruffle structures, was mainly localized at the leading edge of the cells forming large lamellipodia in response to CdtB (Figure 5A and B).

Despite cytoskeleton remodeling, Western blot analysis did not reveal differences in β-actin, α-tubulin, or vinculin expression levels under all the conditions tested (Figure 6A and 6B and data not shown), suggesting that the influence of CdtB was mainly on the localization of these proteins. Nevertheless, cortactin expression increased significantly in response to CdtB in coculture and transduction experiments at both the gene and protein levels (Figure 6). On the basis of these results, the effects of CdtB were evaluated on cellular adherence. The PV-CdtB–transduced cells presented a significant reduced number of adherent cells after 6 hours as compared to the control or PV-GFP-transduced cells (Figure 7). These results suggest that an alteration of the cytoskeleton and focal adhesion by CdtB modifies some cellular functions and particularly those linked with epithelial adherence.

**DISCUSSION**

*H. pullorum*, a bacterium to which humans are exposed via poultry consumption, is an emerging pathogen in digestive diseases that has been little studied [1–13]. In the present study, the effects of 10 *H. pullorum* strains were evaluated using 3 intestinal epithelial cell lines. A similar cytopathogenic effect, mediated by a secreted factor, was observed for all of the strains independently of their human or avian origin in Caco-2 and

**Figure 3.** Detection and effects of CdtB expression in human intestinal epithelial cell line Caco-2. The data were obtained after 72-hour transduction of Caco-2 cells without (control) or with the lentivirus particles used to express the enhanced green fluorescent protein (PV-GFP), or the lentivirus particles used to express the CdtB fused to 3 repeats of the human influenza virus hemagglutinin (HA) epitope (PV-CdtB). A. Western blot analysis of Caco-2 cells. α-tubulin (50 kDa) was used as a reference protein and was detected under all the conditions tested. The GFP (27 kDa) and HA epitope expression (34 kDa corresponding to the expected Mr of CdtB fused to 3 repeats of the HA epitope) was detected only in cells transduced with PV-GFP and PV-CdtB, respectively. B. Fluorescence staining of Caco-2 cells. Cells were processed for fluorescence staining with fluorescent-labeled phalloidin to detect F-actin (red) and with the Hoechst 33342 compound to detect the nucleus (blue). Primary anti-HA antibody followed by fluorescent labeled-secondary antibodies (green) was used to detect the HA-tagged CdtB in the PV-CdtB–transduced cells, and the natural fluorescence of the GFP was used in the PV-GFP–transduced cells. Boxes correspond to enlargement of actin ruffles containing the HA-tagged CdtB. Arrows 1, 2, and 3 indicate CdtB-3HA in the nucleus, the cytoplasm, and the lamellipodia at the front edge of the cell, respectively. Scale bar, 10 µm.
Figure 4. Effects of CdtB on human intestinal epithelial cell morphology. The effects were determined 72 hours after the transduction of Caco-2 and HCA-7 cells without (control) or with the lentivirus particles used to express the enhanced green fluorescent protein (PV-GFP), or the lentivirus particles used to express the CdtB fused to 3 repeats of the human influenza virus hemagglutinin epitope (PV-CdtB). A, Cellular morphological changes of Caco-2 (top line) and HCA-7 (bottom line) cells revealed by May Grünwald-Giemsa staining. Arrows indicate enlarged Caco-2 and HCA-7 cells with distended or multiple nuclei. Scale bar, 50 µm. B, Percentage of cells displaying a distended phenotype (ie, those with enlarged or multiple nuclei). C, The cellular proliferation was determined after 48 hours (for HCA-7) or 72 hours (for Caco-2) of transduction. D, Analysis of DNA contents of Caco-2 and HCA-7 cells. Cells were fixed and labeled with DAPI (4′,6′-diamidino-2-phenylindol) and analyzed by flow cytometry. Data represent 1 representative experiment of 3. B and C, white and grey bars represent untransduced cells (control) or cells transduced with PV-GFP or PV-CdtB, respectively. Data represent the mean of triplicates in 1 representative experiment of 3. The discontinuous line shows the basal rate in control cells. *P<.05 vs control.
HCA-7 cell lines, characterized by the formation of nuclear distended giant cells and an arrest of the cell cycle during the G2/M phase. Similar effects were reported for other CDTs of gram-negative bacteria, such as *Helicobacter* species and *Aggregatibacter actinomycetemcomitans* [17, 20, 29]. However, *H. pullorum* did not induce the cytopathogenic effects on the HT-29 cell line. Ceelen et al also reported that some cell lines were not very susceptible to the toxin produced by *H. pullorum* strains [26]. Use of a *cdtB* KO strain of *H. pullorum* yielded no cytopathogenic effects, suggesting that the cytopathogenic effects of *H. pullorum* were due only to the CDT, not to the unidentified soluble cytotoxic factor suspected to be different from the CDT.
or that the effects of this soluble cytotoxic factor are mediated by the CDT, as proposed by Young et al for that of *H. hepaticus* [20].

Research concerning the CDT and especially its active CdtB subunit is hampered by many obstacles, mainly the difficulty to produce and purify sufficient amounts of the 3 subunits to reconstitute a complete toxin, CdtB being toxic for numerous cellular and cell-free expression systems. Others strategies have been used, like microinjection [30–33] or electroporation [34] of the purified CdtB subunit in cells. A transient expression of

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**Figure 6.** Analysis of the effects of CdtB on cortactin expression. Western blotting analysis of cortactin expression in CaCo-2 cells after 72-hour coculture (A) or transduction (B) with subsequent quantification of cortactin protein expression. The relative expression rate of cortactin proteins in cells was determined by densitometric analysis of the Western blotting autoradiographs and normalized relative to values for the reference protein, actin. Data represent the mean of 4 independent experiments. C, Expression of the cortactin gene in intestinal epithelial cell lines Caco-2 and HCA-7 after 72-hour incubation without or with lentivirus particles. The expression of the cortactin gene in cells was measured by real-time polymerase chain reaction and normalized relative to expression of the reference gene, hypoxanthine phosphoribosyltransferase 1. Results are the means of 3 independent experiments, each performed in triplicate. Ratios were calculated using the \( \Delta \Delta \text{Ct} \) method. The relative expression rate of the cortactin protein and gene was reported as a fold increase versus expression in control cells cultured without *H. pullorum* (A) or without lentivirus particles (B and C). The discontinuous line shows the basal rate of cortactin expression by control cells. \( *P < .05 \) versus control. Abbreviations: \( \Delta \text{cdtB} \), cdtB isogenic mutant of *H. pullorum* strain CCUG 33840; mRNA, messenger RNA; PV-GFP, lentiviral particles used to expressed the enhanced green fluorescent protein; PV-CdtB, lentiviral particles used to expressed the CdtB fused to three repeats of the HA epitope; WT, wild-type *H. pullorum* strain CCUG 33840 (avian origin).
CdtB was successively achieved by gene delivery systems, using plasmids in transfection and lipofection experiments [30, 35]. Only one study reported an adenovirus-based stable expression system of CdtB from *H. ducreyi* [36]. Unfortunately, expression of CdtB was minimal or undetectable in these systems. An alternative is to use another type of expression system, such as lentivirus vectors, since they can transduce a wide range of cell types and integrate into the host genome in both dividing and postmitotic cells, resulting in stable expression of the transgene.

We initially attempted to develop an inducible lentivirus-based expression system [37] to produce CdtB under the control of the EF1-α promoter inducible by tetracycline (a Tet on/off system using the tTR-KRAB repressor). However, no CdtB-inducible cell line could be obtained, probably because the lack of full repression of the promoter by the tTR-KRAB protein in the absence of tetracycline led to CdtB expression that, although slight, was sufficient to inhibit cell proliferation. Thus, new transductions were performed for each experiment, requiring regular production of lentiviruses. However, in contrast to the adenovirus CdtB-based expression system previously reported [36], the high efficiency of this lentivirus transduction system allowed the detection of CdtB by Western blot and also by immunofluorescence at its expected site (ie, the nucleus). The phenotypes induced by this eukaryotic recombinant CdtB were similar to those induced by the *H. pullorum* WT strain, validating the use of the lentivirus expression system. In addition, the effects observed using the lentivirus particles were more pronounced than those observed in coculture experiments, thus facilitating the observation and the study of CdtB effects. This is due to the constitutive and stable expression of CdtB in transduced cells and to the high percentage of transduced cells (approximately 90%). In fact, the effects observed in coculture experiments may be underestimated because of the low survival of *H. pullorum* strains. The higher frequencies of cells blocked during the G2/M phase after the constitutive expression of CdtB in the cells support this hypothesis.

In fact, this new approach, using the lentivirus-based strategy, offers the possibility to overcome technological barriers and represents a new way to explore the role of CdtB toxin of *H. pullorum*.

Interestingly, this lentivirus-based expression system allowed the observation of CdtB-associated phenotypes in the HT-29 cell line, while no cytopathogenic effect was observed when using *H. pullorum* stains. The fact that the HT-29 cell line became susceptible to CdtB by using direct expression of the toxin in the cell suggests that this cell line could lack the CDT receptor (not yet characterized), which would be present in some epithelial cell lines and absent in others, or that one of the pathways required for the internalization or traffic of CdtB, a crucial step for implementing its genotoxic activity [38], is defective, thus rendering the cell resistant to the effects of CdtB. The nuclear localization of CdtB observed in HT-29 cells after transduction with lentivirus particles suggests that, once inside the cytosol, the transport of the toxin into the nucleus is not affected.

In the present study, the CdtB of *H. pullorum* induces the following major effects on the cytoskeleton of intestinal...
epithelial cells: formation of large lamellipodia structures enriched in cortical actin, relocation of vinculin from focal adhesion sites to the perinuclear region of the cytosol, and upregulation of cortactin expression and a decreased cellular adherence. No regulation of β-actin expression was observed either for the CDT or \textit{H. pullorum}. The lack of regulation of some cytoskeletal proteins by \textit{H. pullorum} was previously observed for 2 common target proteins of bacterial pathogens, β-actin and α-tubulin \cite{14, 28}. Nevertheless, a possible effect on the localization of these proteins should not be excluded, as for other bacteria \cite{39}. The effects on cortactin, a central regulator of the actin cytoskeleton, is not surprising, because cortactin is generally considered to promote cell migration by controlling leading-edge lamellipodial dynamics and is one of the targets favored by the pathogen \cite{40}. The formation of lamellipodia structures in response to CdtB was observed in the 3 intestinal epithelial cell lines tested. This effect is in agreement with the recent results obtained in vitro with \textit{H. pullorum} strain 6350–92 (CCUG 33838), which showed that \textit{H. pullorum} can adhere to Caco-2 cells through a flagellum-microvillus interaction that causes membrane ruffling \cite{16}. Another bacterial protein, \textit{Citrobacter rodentium} EspT, triggers formation of lamellipodia on fibroblasts and membrane ruffles on epithelial cells, with these processes dependent on activation of Rac1 and Cdc42 Rho GTPases \cite{41}. These effects are very similar to those observed in the present model in response to \textit{H. pullorum} CdtB, and a possible involvement of \textit{H. pullorum} CdtB in Rac1 or Cdc42 activation should be investigated.

In conclusion, \textit{H. pullorum} is responsible for a cytotoxic disintegrating phenotype on Caco-2 and HCA-7 intestinal epithelial cells, inhibition of cellular proliferation via a cell cycle arrest in the G2/M phase, a profound remodeling of the actin cytoskeleton with the formation of lamellipodia, and a modulation of the cortactin expression. Disturbance of focal adhesion and the microtubule network were also observed. These effects have functional consequences because they are associated with a reduced capacity for adherence. They are due to CdtB and seem to be independent of the human or avian origin of the strain. The profound changes observed on cellular cytoskeleton structures of intestinal epithelial cell lines strengthen the hypothesis that \textit{H. pullorum} infection, via CdtB activity, strongly affects the intestinal barrier integrity.

CDT is secreted by numerous enteropathogens that cause acute diarrheal diseases or are suspected to be involved in the development of liver or inflammatory bowel disease. Depending on the colonized organ, these bacteria could induce, via CDT activity, an inflammatory effect on the epithelial cells \cite{42} and could impair mucosa in the susceptible host. Indeed, \textit{C. jejuni} CDT is diarrheagenic in a dose-dependent manner and induces tissue damage in suckling mice \cite{43}. With regard to \textit{H. pullorum}, the bacterium presents a tropism for the duodenum, cecum, liver, and gallbladder of gallinaceous birds \cite{1, 44} and humans \cite{1–13}, suggesting that \textit{H. pullorum} CDT could mainly affect these enterohepatic ducts. Moreover, studies have shown that chronic infection by CDT-producing bacteria might promote malignant transformation and cancer \cite{25, 45}. However, the exact role of CDT in the pathogenesis of digestive diseases and cancer remains to be elucidated.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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The sequences of \textit{H. pullorum} strains H152 (CCUG 33837), 459–94 (CCUG 33840), 3758–94 (CCUG 33842), H342 (NCTC 13153), H344 (NCTC 13154), 6350–92 (CCUG 33838), 3166–93 (CCUG 33839), H418 (NCTC 13155), H437 (NCTC 13156), and H443 (NCTC 13157) are available in the GenBank database under the accession numbers JX434690 to JX434699, respectively.

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