Evolution of the caecal epithelial barrier during Clostridum difficile infection in the mouse

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SUMMARY   The most striking effect of *Clostridium difficile* infection is its degrading of the intestinal barrier. The aim of this study is to establish whether the cellular or paracellular constituent of the barrier is the initial target of the toxins produced by *C difficile*. Accordingly, the caecal epithelium of C3H/He mice was challenged under three experimental conditions with the *C difficile* strain VPI 10463: (1) by *in vivo* inoculation of axenic mice, (2) by adding the toxins to ligated caeca *in vivo*, and (3) by adding them to the mucosal side of isolated caeca in Ussing chambers. Under all three conditions, the epithelial barrier was tested in caeca mounted in these chambers. The transepithelial potential difference (PD), electrical conductance (G), and intact and degraded Horseradish peroxidase (HRP) fluxes were used as indexes of permeability. Results were as follows: (1) In axenic mice, *C difficile* caused severe infection, produced toxins A and B, reduced PD, and enhanced G and intact HRP fluxes without changing degraded HRP fluxes, (2) four hours after the toxins were added to ligated caeca *in vivo*, PD was relatively unaltered, but G, and intact and degraded HRP fluxes increased, and (3) when toxins were added to caeca during two hours in the Ussing chambers, the only modification observed was an increase in degraded-HRP fluxes. These results indicate that the *C difficile* toxins gradually cause intestinal lesions. After an apparent resistance, they stimulate the endocytotic process and then increase paracellular permeability and finally cause loss of cell viability.

Pseudomembranous colitis in human and in experimental animal models is known to be caused by *C difficile* multiplication in the digestive tract.[1][2] Gnotobiotic mice constitute a good experimental model for reproducing this pathology because their histopathological lesions are similar to those observed in man[3]; they die within two days of infection and large amounts of toxins A and B are detected in their caecal contents.[4] Different studies have shown that toxin A (enterotoxin) induced diarrhoea and that toxin B (cytotoxin) was responsible for the observed haemorrhages; both toxins kill the mice.[5] This model therefore appears to be of interest for studying the relationship between the time course of the infection, the production of toxins and their effect on intestinal function. In the present study, the gradual response of the caecum to infection was tested by *in vitro* measurement in

Ussing chambers. We measured the transepithelial potential difference as an index of tissue viability, transepithelial electrical conductance as an index of paracellular integrity[6] and transepithelial HRP degradation as an index of cellular activity, including endocytosis and lysosomal degradation.[7]

Methods

INFECTIOUS AGENT: C DIFFICILE STRAIN

Strain VPI 10463 of *C difficile* isolated from human tissue[8] was grown in an anaerobic chamber using brain heart infusion broth (BHI, Difco, Detroit, USA).[9]

QUANTIFICATION AND PRODUCTION OF TOXINS

Toxin A was quantified by a previously described immunoenzymatic assay.[10] Its concentration was expressed in log 10 ng/g of caecum or per millilitre of culture broth. The toxin B titre was measured in a cell line of Chinese hamster ovary cells (CHO-K1),[4] and

corresponded to the $\log_{10}$ of the highest dilution which induced a cytotoxic effect in our test.

A crude toxin preparation was obtained using *C difficile* grown inside dialysis bags in flasks containing autoclaved BHI. Flasks were incubated for four days at 37°C in an anaerobic chamber. The amounts of toxin A in the supernatant of the dialysis bag and the toxin B titre were 6·5 and 4 respectively. This preparation was used in the Ussing chambers.

EXPERIMENTAL SCHEDULE

All the mice were of the C3H/He strain and were fed a commercial diet *ad libitum* (RO3-40, UAR, Villemoisson, France). Axenic mice were reared in Trexler-type isolators fitted with a rapid transfer system (La Cahlène, France). All items used for these mice were sterilised by heat or gamma irradiation. Holoxenic mice were derived from axenic mice and were kept in a clean but not sterile environment.

GROUP 1: MICE MONOASSOCIATED WITH C DIFFICILE

Axenic mice were inoculated through the orogastric route with 1 ml of a 24 h culture of *C difficile* (10^8 vegetative cells/ml). Under these conditions, they developed pseudomembranous colitis characterised by intense caecal abrasion together with a severe inflammatory process; all the animals died within two days of infection.[4] At various times after infection, mice were killed and their caecum was removed. Some caeca were used in Ussing chambers and others, to determine toxin B titres and the amounts of toxin A.

GROUP 2: HOLOXENIC MICE WITH TOXINS INJECTED INTO LIGATED CAECA IN VIVO

Holoxenic mice were anaesthetised by a 0·2 ml intraperitoneal injection of 0·6 mg/ml pentobarbital. After laparotomy, the distal part of the ileum and the proximal part of the colon were exposed and ligated separately taking care to avoid any constriction of vessels. 0·1 ml toxins or BHI broth were then injected into the upper part of the caecum; this portion was not used in the Ussing chamber. Animals were then sutured and allowed to recover. Mice injected with toxins died in about four hours. At that time, abrasion and inflammation of the caecal mucosa were noted. Some of the mice in this group were killed just before they died of the effects of the toxins and only haemorrhagic caeca (80%) were used in the Ussing chambers. Control mice with ligated caeca survived for one day after surgery. Some of these mice were killed four hours after the surgical procedure but their transport characteristics were identical to those of control mice without previous surgery. Axenic mice were not used in these experiments because they did not survive this surgical procedure.

GROUP 3: CAECAL EPITHELIUM OF HEALTHY MICE MOUNTED IN USSING CHAMBERS IN THE PRESENCE OF TOXINS.

Caeca were collected from the axenic and holoxenic mice. They were used in Ussing chambers containing the same amount of toxins as the caeca of groups 1 and 2.

MEASUREMENT OF THE EPITHELIAL BARRIER FUNCTION

The treated caecal tissues of all the groups of mice, were mounted in Ussing chambers and the capacity of the epithelium to maintain its barrier function was assessed by measuring electrical conductance, an index of paracellular permeability,[6] and transport of HRP in an intact or degraded form, used as an index of endocytosis and lysosomal degradation by the cells.[7]

The caeca were opened as flat sheets, rinsed with cold Ringer solution and placed on Millipore filters (HAMK 0·45 µm). The filters and tissues were mounted in small Ussing chambers as previously described.[11] The exposed area was 15 mm². Silicone paste was used to minimise edge damage. Both sides of the tissues were bathed at 37°C with 2 ml of Ringer solution (pH=7·4) which was oxygenated (95% O_2, 5% CO_2) and supplemented with 10 mM glucose and 10 mM glutamine. The solutions bathing the mucosal – that is, luminal – and serosal – that is, blood – sides of the tissues were connected *via* 3 M KCl-agar bridges to calomel electrodes for measurement of the transepithelial potential difference (PD) and to Ag-AgCl electrodes for the passage of current through the system. Open circuit conditions were used in flux experiments. Tissue conductance (G) was assessed regularly by passing a 10-µA current through the preparation. HRP (Sigma type VI) at a final concentration of 10 µM (0·4 mg/ml) and 200 µl of crude *C difficile* toxins (toxin B titre 6·5 and toxin A at 4 $\log_{10}$ ng/ml) were added to the mucosal compartment. Tritiated-HRP (3H-HRP), prepared and stored as previously reported,[7] was also added to the mucosal compartment as a tracer (1·35 µCi). Over a period of 130 min, 1·3 ml samples were taken from the serosal compartment at 10 and 20 min intervals and replaced by fresh buffer. The rate at which intact HRP was transferred to the serosal side was determined by enzymatic assays on 200-µl aliquots according to the modified Worthington method.[12] Fluxes were expressed in pmoles/h/cm². Total HRP transport – that is, intact plus degraded – was assessed on 500-µl aliquots by tritium counting, using liquid scintillation photometry. Degraded HRP fluxes
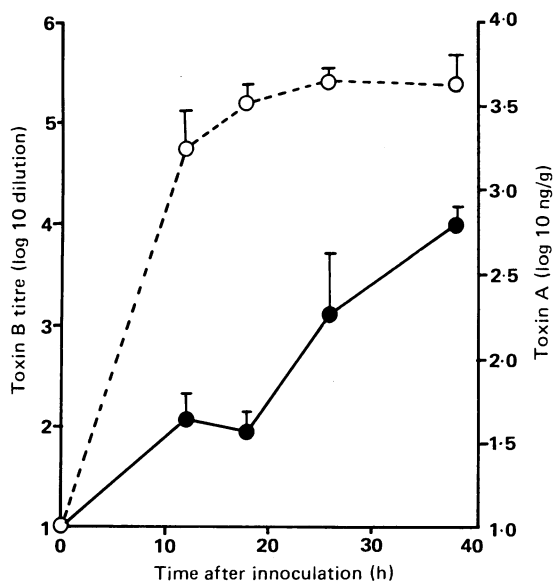
Fig. 1 *Kinetics of toxin A (●) and B (○) production during experimental infection of axenic mice with* C difficile.

(J degraded HRP) were calculated as total minus intact HRP fluxes (J intact HRP).

STATISTICAL ANALYSIS
Means (SE) were compared using Student's *t* test and variance analysis.

Results

EFFECT OF C DIFFICILE INFECTION IN AXENIC MICE (GROUP 1)

Production of toxins
In axenic mice, *C difficile* was established very soon after inoculation; the maximal number (10^8/g of

Table 1 *Effect of* C difficile *infection on the caecal epithelial barrier of axenic mice, at 26 and 38 h post infection*

	Axenic control mice	Axenic infected mice	
		26 h pi	38 h pi
PD (mV)	1·02 (0·20)	1·23 (0·58)	0·20 (0·08)*
	(n=6)	(n=4)	(n=6)
G (mS/cm²)	27·06 (3·15)	58·4 (30·0)	45·7 (9·6)
	(n=6)	(n=4)	(n=6)
J intact HRP	0·20 (0·15)	4·52 (0·41)	7·58 (1·96)†
(pmol/h/cm²)	(n=5)	(n=6)	(n=6)
J degraded HRP	9·88 (2·75)	2·88 (1·91)	4·96 (1·33)
(pmol/h/cm²)	(n=6)	(n=6)	(n=6)

Significantly different from controls: *p<0·05; †p<0·01; pi=post infection.

caecum) was reached 12 h thereafter (data not shown). At this time, toxin B production was near its maximum (Fig. 1) but only small amounts of toxin A were detected. Two groups of mice were used in the Ussing chamber experiments. The first one was killed 26 h post infection (pi), when the toxin B titre was 5·2 and the $\log_{10}$ ng/g of toxin A was about 2·3. The second group of animals was killed 38 h post-infection, when the toxin B titre was at about the same level, but the amount of toxin A had reached its maximum. Unlike the animals killed at 26 h, those killed at 38 h were dying and their caeca were haemorrhagic.

MEASUREMENTS OF THE EPITHELIAL BARRIER AFTER 26 AND 38 H OF INFECTION
At 38 h post infection, the potential difference (PD) in infected mice was significantly less than in control axenic mice (Table 1). At 38 and 26 h post infection, ionic conductance (G) tended to increase although not significantly. G was increased in one in four animals at 26 h post infection and four of six animals at 38 h post infection. Degraded HRP fluxes, which are necessarily transcellular, were not significantly different from those of the control group, even though transcellular transport tended to decrease. Both after 26 and 38 h post infection, the intact HRP fluxes gradually increased compared with those observed in control axenic mice. The difference only became significant after 38 h post infection (p<0·01). In the latter case, intact HRP transport increased linearly as a function of time, indicating tissue leakage, whereas in control caeca, the transport remained saturable, indicating that its route was transcellular. Note that ionic conductance (G) and intact HRP fluxes both increased in the infected mice, and that these two parameters were significantly correlated (p<0·05).

Measurement of toxin transport
Two hundred microlitres of crude extract containing toxins A and B was placed in the mucosal reservoir of the Ussing chambers during the HRP flux measurements. Serosal samples taken at 20 min intervals were assayed for toxin A and toxin B activity. Neither toxin was detected in these samples, suggesting that very little or no toxin crossed the caecal epithelium.

MEASUREMENT OF THE EPITHELIAL BARRIER AFTER IN VIVO INJECTION OF CRUDE TOXINS INTO LIGATED CAECA (GROUP 2)
To examine the role of toxins in damaging the caecal epithelium during infection, caecum was ligated and exposed to a crude toxin extract for 4 hours *in vivo*.
After a four hours of contact, animals were killed and the caecal tissue was excised and mounted in an
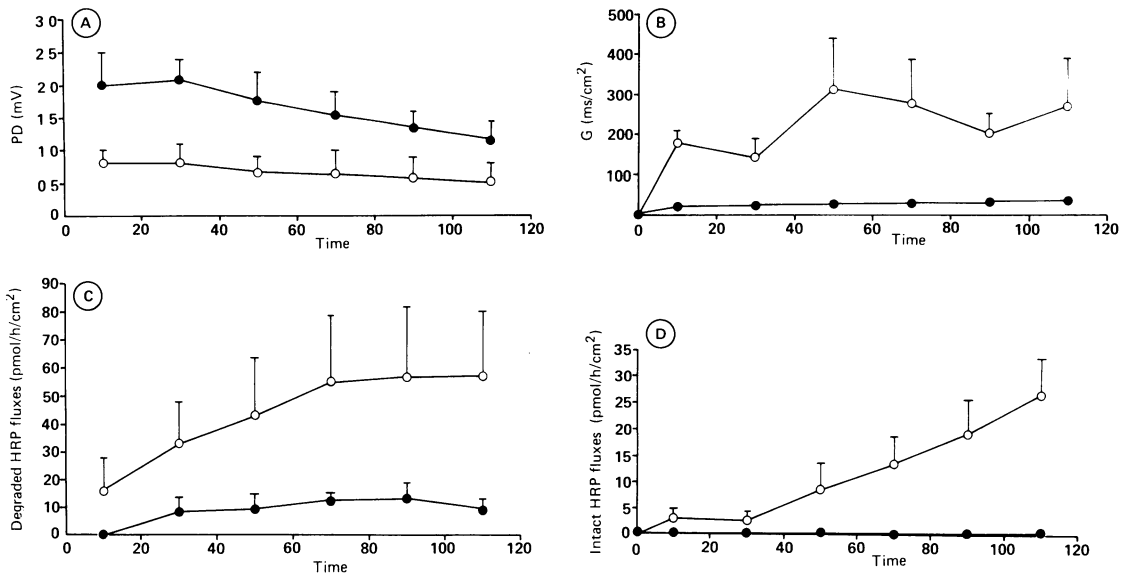
Fig. 2   *Transepithelial potential difference (A) ionic conductance (B), degraded HRP fluxes (C) and intact HRP fluxes (D) during the time course of the experiment in the Ussing chamber, in control (●) and toxin treated (○) animals. Ligated caeca were incubated for four hours* in vivo *in the presence of crude toxins before being mounted in Ussing chambers. Toxins were also present in the mucosal compartment.*

Ussing chamber. The electrical parameters were stable for the period between 60 and 110 minutes thereafter (Fig. 2a, b). The epithelial cells remained functional since the PD, although lower, was not statistically different from that of the control. Toxin treated caeca exhibited a sevenfold increase in ionic conductance, however, compared with control caeca ($p<0.01$). The consequences of this increased conductance were explored by measuring intact HRP and toxin fluxes. Intact fluxes increased linearly as a function of time (Fig. 2d) and after 50 minutes in the Ussing chamber, were significantly different from

control fluxes ($p<0.01$). Neither toxin A or B fluxes, however, were detectable at any time. A correlation analysis of both the intact HRP fluxes and G in tissue from control and treated mice showed that these two parameters were significantly correlated ($p<0.001$), which suggests paracellular leakage. Furthermore, the degraded-HRP fluxes (Fig. 2c) rose significantly compared with controls ($p<0.05$), suggesting that the transcellular endocytosis was maintained and even stimulated.

MEASUREMENT OF THE EPITHELIAL BARRIER FOR TWO HOURS IN THE PRESENCE OF CRUDE TOXINS IN USSING CHAMBERS (GROUP 3)

To identify the initial target of the toxins on the epithelium, control caeca were mounted in Ussing chambers and tested *in vitro* for two hours in the presence of crude toxins.

As steady state values were obtained for HRP fluxes after 60 minutes, the values reported in Table 2 are the means of the three last measurements, made between 60 and 130 minutes. The presence of toxins did not alter the electrical parameters PD or G. Intact HRP fluxes remained similar in the presence and absence of toxins. A significant increase in degraded HRP fluxes was noted in the caeca of axenic mice, however, suggesting a rise in endocytosis. In no case did we detect the presence of toxin A or B in the serosal reservoir of the Ussing chambers.

Table 2   *Effect of crude toxins of* C. difficile *on the caecal epithelial barrier after two hours in Ussing chambers*

	Axenic Mice		Holoxenic mice	
	Control caecum	Caecum with toxins	Control caecum	Caecum with toxins
PD (mV)	1·02 (0·20)	1·57 (0·57)	1·32 (0·33)	1·58 (0·53)
	(n=6)	(n=6)	(n=8)	(n=6)
G (mS/cm²)	27·06 (3·15)	25·95 (3·24)	32·75 (6·00)	33·56 (3·60)
	(n=6)	(n=5)	(n=6)	(n=8)
J intact HRP	0·20 (0·15)	0·19 (0·12)	0·25 (0·10)	0·54 (0·22)
(pmol/h/cm²)	(n=5)	(n=4)	(n=6)	(n=6)
J degraded HRP	9·88 (2·75)	21·94 (6·86)*	10·38 (4·23)	11·17 (2·14)
(pmol/h/cm²)	(n=6)	(n=4)	(n=6)	(n=6)

*Significantly different from the other groups $p<0.05$.

Intact HRP transferred (%)

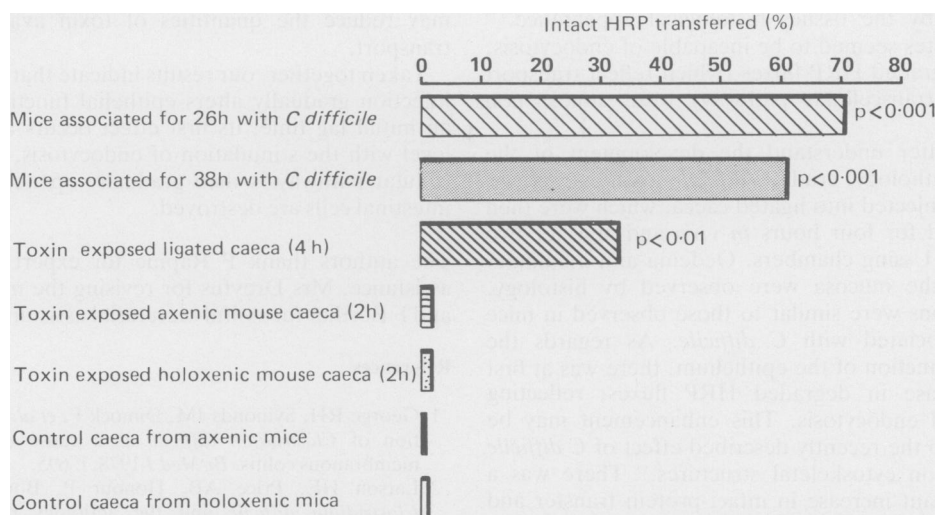|	0 10 20 30 40 50 60 70 80
Mice associated for 26h with *C difficile*	p<0·001
Mice associated for 38h with *C difficile*	p<0·001
Toxin exposed ligated caeca (4 h)	p<0·01
Toxin exposed axenic mouse caeca (2h)	
Toxin exposed holoxenic mouse caeca (2h)	
Control caeca from axenic mice	
Control caeca from holoxenic mica	

Fig. 3 *Percentage of intact HRP transferred from mucosa to serosa (total transfer=intact+degraded HRP transfer=100%) across caeca treated with* C difficile *toxins under different experimental conditions. Results for the first three series of caeca are significantly different from those for the corresponding control caeca.*

As regards the percentage of intact HRP transported under the different experimental conditions (100% representing total transport), Figure 3 shows that with time, the action of the toxins progressively increased intact HRP transport, reflecting gradual mucosal damage.

Discussion

The present results confirm that *C difficile* infection in mice causes intestinal lesions, probably as a result of the action of its toxins, and that these events finally lead to systemic effects and death. They also show the gradual action of the toxins on epithelial function. After an apparent initial resistance, an increase in the endocytosis of macromolecules is rapidly followed by increased paracellular permeability and finally loss of cell viability.

Physiological endocytosis of macromolecules has been reported to have no pathological implications,[13] and previous work from this laboratory[7 11] showed the usefulness of the HRP marker for studying protein transport across the intestine. Measurement of degraded HRP fluxes reflected endocytosis and lysosomal degradation by the enterocytes. Under normal conditions, 90% of the protein is degraded during transport, and the remaining 10% is transported intact via a pathway that is probably also transcellular. In the present work, HRP was used as a marker of epithelial function and integrity throughout the time course of an intestinal infection with *C difficile*.

Our experimental conditions were chosen in order to have different periods of contact between *C difficile* toxins and the caecal epithelium – that is, two hours contact in an Ussing chamber, four hours contact in ligated caeca followed by Ussing chamber measurements, and longer contact during experimental infection. All experiments were carried out with the same doses of toxins. In Ussing chambers and ligated caeca, the amounts of toxin were adjusted by diluting the crude extract so as to obtain the same amounts as the maxima measured *in vivo* in infected mice.

The present study allowed examination of the electrical parameters in the course of infection. Previous studies showed the effects of toxins A and B on the electrogenic properties of rabbit intestinal tissues mounted in an Ussing chamber.[14–16] The present results did not show any electrogenic secretion at the caecal level after two hours of contact with the toxins. A large increase in ionic conductance, was only detected after four hours of contact with the toxins, indicating possible paracellular disruption.

In rodents, it is well known that toxins A and B are produced together.[17] Our results indicate that the kinetics of the production of the two toxins are different. The fact that death occurred when toxin A production was maximal suggests that this toxin is necessary to cause death. Toxin B production was almost maximal at about 10 h postinfection, when toxin A had only just begun to be produced. In the group of mice monoassociated with *C difficile*, transport of intact protein through the caeca and ionic conductance were enhanced, suggesting epithelial leakage. Disruption of the epithelial layer was also

attested by the tissues histological appearance.[3][18] Enterocytes seemed to be incapable of endocytosis, since degraded HRP fluxes (which reflect transport *via* the transcellular pathway) were almost non existent.

To better understand the development of the caecal pathology, crude *C difficile* toxin extract was directly injected into ligated caeca, which were then incubated for four hours *in vivo* and another two hours in Ussing chambers. Oedema and inflammation of the mucosa were observed by histology. The lesions were similar to those observed in mice monoassociated with *C difficile*. As regards the barrier function of the epithelium, there was at first an increase in degraded HRP fluxes, reflecting enhanced endocytosis. This enhancement may be related to the recently described effect of *C difficile* toxin B on cytoskeletal structures.[19] There was a concomitant increase in intact-protein transfer and total ionic conductance indicating gradual disruption of the caeca. It is possible that an opening of the tight junctions was responsible for the passage of the intact HRP.[20] The lack of saturation of intact HRP fluxes as a function of time indicates leakage through the disrupted epithelial layer. Transcellular passage would have led to steady state fluxes, as previously shown.[6] In addition, there was a significant correlation between intact HRP fluxes and G, suggesting that they used a common paracellular pathway.

In the epithelial barrier measurements on caeca exposed to toxins for two hours in Ussing chambers, no histological damage was observed, a result which agreed well with the stability of ionic conductance an index of paracellular integrity. Transport of intact HRP was not modified in axenic or holoxenic mice. Endocytosis, as judged by the degraded HRP-fluxes in axenic animals, was stimulated by the presence of toxins. This effect was also observed in holoxenic mice, but only after four hours of exposure. These observations indicate that the endocytotic capacity of the enterocytes may increase after a short contact with *C difficile* toxins. They also indicate initial resistance of the tissue to the toxins possibly due to the mucus layer acting as a protectant.

The question of whether or not toxins are absorbed across the intestinal epithelium remains unsolved. Assuming that the efficiency of toxin transport is similar to that observed for HRP (ratio transported in one hour: $1/25000$), the present immunoenzymatic assay is not sensitive enough to detect a transport. According to our assays sensitivity, it is possible to suggest that less than $1/10000$ of cytotoxic activity and less than $1/1000$ enterotoxic activity is transported and perhaps none at all. Furthermore, the existence of a receptor on the brush border membrane of the intestine, and the binding of toxin A to enterocytes[21]

may reduce the quantities of toxin available for transport.

Taken together, our results indicate that *C difficile* infection gradually alters epithelial function. After an initial lag time, its first effect occurs at cellular level with the stimulation of endocytosis, the paracellular pathway is then gradually opened and the intestinal cells are destroyed.

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