Pet secretion, internalization and induction of cell death during infection of epithelial cells by enteroaggregative *Escherichia coli*

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In an *in vitro* model using HEp-2 cells treated with purified plasmid-encoded toxin (Pet), we have identified morphological changes characterized by cell rounding and detachment after toxin internalization; these changes progress to cell death. However, these effects have not yet been shown to occur during the infection of epithelial cells by enteroaggregative *Escherichia coli* (EAEC). Here, we show that the secretion of Pet by EAEC is regulated at the transcriptional level, since secretion was inhibited in eukaryotic cell culture medium, although Pet was efficiently secreted in the same medium supplemented with tryptone. Inefficient secretion of Pet by EAEC in DMEM prevented cell detachment, whereas efficient Pet secretion in DMEM/tryptone increased cell detachment in a HEp-2 cell adherence assay. Interestingly, Pet toxin was efficiently delivered to epithelial cells, since it was internalized into epithelial cells infected with EAEC at similar concentrations to those obtained by using $37 \mu\text{g ml}^{-1}$ purified Pet protein. Additionally, Pet was not internalized when the epithelial cells were infected with a *pet* clone, HB101(pCEF1), unlike the wild-type strain, which has a high adherence capability. There is a correlation between Pet secretion by EAEC, the internalization of Pet into epithelial cells, cell detachment and cell death in EAEC-infected cells. The ratio between live and dead cells decreased in cells treated with wild-type EAEC in comparison with cells treated with an isogenic mutant in the *pet* gene, whereas the effects were restored by complementing the mutant with the *pet* gene. All these data indicate that Pet is an important virulence factor in the pathogenesis of EAEC infection.

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INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) is an enteropathogen responsible for persistent diarrhoea in children from developing and industrialized countries, as well as for traveller's diarrhoea (Harrington *et al.*, 2006). The diarrhoea induced by EAEC is characterized by persistent liquid evacuation and abundant mucus secretion, frequently accompanied by blood (Nataro *et al.*, 1995). A toxin secreted by EAEC, called plasmid-encoded toxin (Pet), is able to induce enterotoxic and cytotoxic effects in a model of rat intestine preparations mounted in Ussing chambers and in cell cultures (Navarro-Garcia *et al.*, 1998, 1999). Pet is a toxin of 104 kDa encoded on the 65 MDa plasmid AA of EAEC (Eslava *et al.*, 1998). This toxin belongs to the serine protease autotransporter of *Enterobacteriaceae* (SPATE) family, members of which are produced by diarrhoeagenic *E. coli* and *Shigella* species, because Pet possesses a catalytic serine protease motif and uses the type V secretion system, initially described for immunoglobulin-A proteases of *Neisseria* and *Haemophilus*

spp. (Henderson *et al.*, 2004; Jose *et al.*, 1995). The activity of the serine protease motif of autotransporters has been characterized for a number of these proteins by using purified protein assays (Benjelloun-Touimi *et al.*, 1995; Henderson *et al.*, 1999a; Maroncle *et al.*, 2006; Navarro-Garcia *et al.*, 1999, 2004).

The participation of the Pet toxin in damaging the intestinal epithelium is a key event which has been evidenced by *in vitro* organ cultures (Henderson *et al.*, 1999b). *In vitro* studies have also demonstrated that Pet is internalized into epithelial cells by clathrin-mediated endocytosis (Navarro-Garcia *et al.*, 2007b). Once inside the cells in early endosomes, Pet undergoes retrograde transport from the Golgi complex to the endoplasmic reticulum, where it utilizes the Sec61 translocator to reach the cytosol (Navarro-Garcia *et al.*, 2007a). The presence of Pet in the cytosol correlates well with the intracellular colocalization of a target, α -fodrin, which is cleaved by Pet to cause the cytotoxic effect observed in HEp-2 cells treated with purified Pet (Canizalez-Roman & Navarro-Garcia, 2003). The observed damage in HEp-2 and HT29 cells is associated with cytoskeleton contraction, loss of actin stress fibres and release of the focal contacts, followed by cell

Abbreviations: EAEC, enteroaggregative *Escherichia coli*; Pet, plasmid-encoded toxin; SPATE, serine protease autotransporter protein.

rounding, detachment and death (Navarro-Garcia *et al.*, 1999). All these intracellular effects depend on the serine protease activity of Pet (Canizalez-Roman & Navarro-Garcia, 2003; Navarro-Garcia *et al.*, 1999).

So far, it is clear that purified Pet follows a sequence of events in order to damage the epithelial cells. However, all these events have been deduced from research performed in *in vitro* systems using purified Pet toxin ($37 \mu\text{g ml}^{-1}$) and have not yet been shown to occur during the infection of epithelial cells by EAEC. Here, we designed an *in vivo* infection model using EAEC bacteria and HEp-2 cell cultures. We examined the secretion of Pet by EAEC in diverse media, the efficiency of toxin internalization in our infection model, and finally we performed a qualitative and quantitative analysis of morphological changes and cell death caused by Pet secreted during infection with EAEC. Our results showed that an efficient secretion of Pet toxin is induced when EAEC is cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with tryptone but not in minimal essential medium (MEM) or DMEM alone. EAEC secretes enough Pet toxin to be internalized by the epithelial cells, an event which depends on another EAEC factor, such as adherence factor. Finally, we found an important correlation between the secretion/internalization of the toxin and the cytotoxic effects and cell death induced by Pet-secreting EAEC.

METHODS

Bacterial strains, plasmids and culture media. *E. coli* 042 wild-type was obtained from a case of infantile diarrhoea caused by EAEC in Lima, Peru (Nataro *et al.*, 1995). The isogenic mutant in the *pet* gene (EAEC $_{pet}^-$) has been previously described (Henderson *et al.*, 1999b). The isogenic mutant was complemented with a plasmid containing the *pet* gene (pCEFN1) encoding the native Pet protein (Eslava *et al.*, 1998). Bacteria were preserved at -80°C and were grown by seeding in Luria-Bertani (LB) agar or LB broth before each experiment.

For Pet secretion assays, the bacteria were grown in the following media: M9 (minimal culture medium for *E. coli*), bacterial enriched culture medium using brain and heart infusion (BHI), pleuropneumonia-like organism (PPLO; enriched medium for culturing *Mycoplasma*), MEM, DMEM and RMPI (enriched medium for human cells). Before each experiment, $100 \mu\text{l}$ bacterial culture were seeded in 3 ml LB and cultured at 37°C with shaking at 150 r.p.m. until the exponential phase.

Cell culture. HEp-2 cells [American Type Culture Collection (ATCC), no. CCL-23] were used as a model, since Pet has the same effects on these cells as on intestinal HT29-C1 epithelial cells, and HEp-2 cells are easier to handle. HEp-2 cells were propagated in humidified 5% $\text{CO}_2/95\%$ air at 37°C in DMEM supplemented with 5% fetal bovine serum (FBS; Hyclone), 1% non-essential amino acids, 5 mM L-glutamine, penicillin ($100 \text{ units ml}^{-1}$) and streptomycin ($100 \mu\text{g ml}^{-1}$). The subcultures were serially propagated after harvesting with 10 mM EDTA and 0.25% trypsin (Gibco-BRL) in PBS (pH 7.4). For experimental use, subconfluent HEp-2 cells were resuspended with EDTA/trypsin, plated onto eight-well LabTek slides (VWR) or Petri dishes ($60 \times 15 \text{ mm}$), and allowed to grow to 70% confluence.

Toxin treatment. For Pet treatment, Petri dish-cultured HEp-2 cells were incubated with purified Pet protein at $37 \mu\text{g ml}^{-1}$ for 3 h at 37°C (Navarro-Garcia *et al.*, 1999). After Pet treatment, the remaining attached cells on the LabTek slides or Petri dishes were detached by adding 0.25% trypsin and resuspended, together with the detached cells, in 1 ml lysis buffer [0.25 M Tris/HCl, pH 7.5, $50 \mu\text{g ml}^{-1}$ PMSF ml^{-1} , $0.5 \mu\text{g ml}^{-1}$ Complete Solution for Protease Inhibition (Roche), $0.5 \mu\text{M}$ EDTA]. The suspension was subjected to three thermal shocks (5 min on dry ice followed by 3 min at 47°C). Proteins ($50 \mu\text{g}$) were separated on 10% SDS-PAGE and gels were stained with Coomassie blue or were transferred to nitrocellulose membranes for Western blotting.

Cellular infection. HEp-2 cells cultured in DMEM/tryptone (1%) with FBS and antibiotics were washed three times with PBS at 37°C . Culture medium was changed for DMEM/tryptone without FBS and antibiotics, and cells were infected at an m.o.i. of 10 with the different *E. coli* strains for 5 h at 37°C . After infection, HEp-2 cells were washed three times and then incubated with fresh medium containing $100 \mu\text{g lysozyme ml}^{-1}$ for 1 h to eliminate the remaining bacteria.

To assay for cell adhesion, monolayers were either fixed, stained with trypan blue and analysed by microscopy, or washed again and detached by adding 0.25% trypsin for further analysis by FACS (see below). Adhesion was determined by microscopic analysis from at least five $\times 40$ fields (~ 300 HEp-2 cells per field) from each of at least six infected monolayers per strain tested.

Pet detection. Pet was precipitated with TCA from supernatants of *E. coli* cultures and from the cytoplasmic fraction of infected cells. Pellets were resuspended in $25 \mu\text{l}$ Tris/HCl, pH 8.8, and $5 \mu\text{l}$ Laemmli sample buffer and separated by 10% SDS-PAGE. Gels were either stained with Coomassie blue or transferred to nitrocellulose membranes. For Western blotting, the membranes were incubated with a rabbit polyclonal antiserum against Pet (1:400) for 1 h, followed by a goat anti-rabbit alkaline phosphatase-conjugated antibody (1:500) for 1 h. The reaction was detected by using the NBT/BCIP substrate (Sigma-Aldrich). Digitized images were analysed by densitometry (SigmaGel).

RT-PCR. RT-PCR was performed using the Reverse-iT 1st Strand Synthesis kit (ABGene) according to the manufacturer's instructions. Briefly, total RNA was extracted from the bacterial cell pellets by using TRIzol reagent. For each reaction, $1.0 \mu\text{g}$ total RNA was used to synthesize the cDNAs by reverse transcription at 47°C for 50 s. Forward and reverse sets of RT-PCR primers were designed as follows: *pet* gene, forward primer 5'-GTG GTG CCT ATG CCG TAA CC-3', reverse primer 5'-CAG CCC CTC TTG TTT CCA CG-3' (which generate a 164 bp PCR product). Samples were subjected to 35 cycles of PCR amplification. In each cycle, denaturing was at 94°C for 60 s, annealing at 55°C for 60 s, extension at 72°C for 60 s and final extension at 72°C for 5 min. The final RT-PCR product was resolved on 1% agarose gels containing ethidium bromide.

FACS assay. For Pet internalization assays, HEp-2 cell pellets were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Washed and centrifuged (500 g) pellets were incubated with rabbit polyclonal antiserum against Pet (1:100) for 30 min; isotype antibodies were included as negative controls. Cells were washed again and the pellets were incubated with goat anti-rabbit FITC-conjugated antibody (1:150) for 20 min. Washed cells (10^4) were analysed by FACS to determine the number of Pet-FITC-positive cells by using a FACSCalibur (Becton Dickinson), and data were processed using CellQuest Software (Becton Dickinson).

For detecting cell death, a suspension of 10^6 Pet-treated HEp-2 cells was stained with acridine orange (1:10 in PBS) and ethidium bromide (1:10 in PBS) for 30 min in darkness. A population of

2×10^4 cells was analysed by FACS for each of the following conditions: at 525 nm for acridine orange and 620 nm for ethidium bromide. To distinguish live and dead cells, WinMDI software was used.

Hep-2 adherence assay. Hep-2 cells were seeded in eight-well LabTek slides (VWR) with DMEM/tryptone and FBS, and allowed to grow to 75 % confluence. Before infection, cells were washed three times with DMEM/tryptone without FBS or antibiotics, and the *E. coli* strains were added at various m.o.i. (as indicated for each assay). After the infection time, cells were washed three times with PBS, fixed with 70 % methanol for 12 min and dried at room temperature for 30 min. Cells were stained with Giemsa for 30 min, and the slides were mounted with resin (Permount) and analysed by light microscopy.

RESULTS

Pet secretion by EAEC 042 in various culture media

In order to select the culture media to produce an *in vitro* infection model, we analysed Pet secretion in different

media. EAEC 042 was cultured in LB and MEM for 2, 4 and 6 h. Pet secretion was detected from TCA-precipitated supernatants by Western blotting. As a positive control, 0.3 μ g purified Pet protein was used (Fig. 1a). In TCA-precipitated supernatants from EAEC culture in LB, a 104 kDa protein band corresponding to Pet protein was observed at 2, 4 and 6 h (Fig. 1a). However, this protein band was not detected in TCA-precipitated supernatants from EAEC cultivated in MEM (Fig. 1a). These data suggest that Pet is not secreted in eukaryotic cell culture media, such as MEM. Therefore, we decided to test other culture media used for eukaryotic or bacterial cells. EAEC was cultured for 18 h in M9, BHI, DMEM, PPLO or RPMI. Only in TCA-precipitated supernatants from EAEC cultured in DMEM was it possible to, barely, detect secretion of Pet (Fig. 1b). A kinetics assay of Pet secretion from EAEC cultured in DMEM (2, 4, 6, 16, 18, 20 and 24 h) showed that Pet was secreted after 6 h of culture; it was less efficient at 16 h than at 18 h and was kept at similar levels at 20 and 24 h (data not shown). In order to

Fig. 1. Pet secretion depends on the culture medium. (a) EAEC 042 secretes Pet in LB medium but not in MEM. EAEC was grown in LB or MEM for 2, 4 and 6 h. Supernatant from culture media was precipitated with 100 % TCA. Total proteins (25 μ g) were separated by 10 % SDS-PAGE and transferred to nitrocellulose membranes. Pet was identified by Western blotting using polyclonal rabbit antibodies against Pet. Antigen-antibody reactions were visualized using alkaline phosphatase-labelled goat anti-rabbit IgG antibodies and developed using NBT/BCIP. (b) Pet is not secreted in various culture media. Pet was precipitated with TCA from supernatants of EAEC grown for 18 h in various culture media: M9, BHI, DMEM, PPLO and RPMI. (c) Pet is efficiently secreted by EAEC in DMEM supplemented with tryptone. Pet was precipitated with TCA from supernatants of EAEC grown for 5 h in MEM, DMEM, DMEM mixed with different ratios of LB, and DMEM supplemented with tryptone. (d) EAEC efficiently grows and secretes Pet in DMEM supplemented with tryptone. Pet secretion was estimated by densitometry alongside an EAEC growth curve (◆) (1–16 h) in DMEM supplemented with tryptone.

increase the secretion of Pet by EAEC in DMEM, EAEC was cultured for 5 h in DMEM mixed with LB medium at various ratios (1 : 2, 1 : 1, 2 : 1), and Pet was analysed in the supernatants. The addition of LB medium to DMEM favoured secretion of Pet by EAEC and this effect was dose-dependent (Fig. 1c). To identify any component from LB medium favouring the secretion of Pet by EAEC cultured in DMEM, EAEC was cultured for 5 h in DMEM supplemented with NaCl, yeast extract (data not shown) or tryptone (Fig. 1c) at the proportions used to formulate LB medium. Only DMEM supplemented with 1 % tryptone (DMEM/tryptone) was able to allow secretion of Pet by EAEC after 5 h of culture; interestingly, none of the previously tested media contained tryptone, except LB medium, and their supplementation with tryptone allowed secretion of Pet.

Finally, to test whether DMEM/tryptone is an adequate culture medium for EAEC cultures, we produced a growth curve of EAEC in this medium. Bacterial growth was detected at 600 nm every hour (up to 16 h) and secretion of Pet was quantified by Western blotting and densitometry

(Fig. 1d). The growth curve showed that EAEC grew (line with diamonds) similarly to how it does in LB medium (not shown), and secretion of Pet was clearly detected in the supernatants after 2 h of culture (continuous line). The secretion of Pet gradually increased up to 15 h and then decayed at 16 h of culture (Fig. 1d). These data indicate that DMEM supplemented with tryptone allows efficient growth of EAEC and enables these bacteria to secrete Pet.

Tryptone causes transcriptional upregulation of Pet expression

To determine whether the increase in secretion of Pet in DMEM/tryptone was related to protein expression or secretion, we decided to detect Pet mRNA in EAEC cultured in different culture media (LB, MEM, DMEM and DMEM/tryptone). Primers for the amplification by RT-PCR of a 164 bp fragment encoding Pet were used, and to analyse constitutive expression, a fragment of 731 bp for *gapA* was amplified. To standardize the detection and specificity of primers, RT-PCR of mRNA from EAEC or an isogenic *pet* mutant (EAEC pet^-) was performed (Fig. 2a).

Fig. 2. Pet secretion by EAEC is inhibited at the transcriptional level in eukaryotic cell culture media. (a) mRNA expression of the *pet* gene by EAEC 042 and an isogenic *pet* mutant. RT-PCR for *pet* (164 bp) and *gapA* (731 bp) gene expression was performed using total mRNA from wild-type EAEC and EAEC 042 pet^- cultured overnight in LB. (b) Kinetics of Pet mRNA expression in various culture media. RT-PCR for Pet mRNA expression by EAEC 042 cultured for 2, 4, 5, 8, 12 or 16 h in LB, MEM, DMEM or DMEM/tryptone. In (a) and (b), RT-PCR for *gapA* gene expression was used as a housekeeping gene control. RT-PCR products were analysed on 2 % agarose gels and bands were visualized by ethidium bromide staining. (c) Densitometric analysis of gel bands for each treatment. Pet mRNA expression was quantified by densitometry as area : intensity ratio ($n=3$).

As expected, the primers amplified expression of Pet mRNA in the wild-type strain by detecting the 164 bp amplicon, whereas this fragment was not detected in samples of mRNA from EAEC pet^- . However, it was possible to amplify the 731 bp fragment for constitutive expression of *gapA* in both mRNA samples (Fig. 2a). This standardized assay was used to analyse the expression of Pet mRNA from EAEC cultured in LB, MEM, DMEM or DMEM/tryptone for various times. It was possible to amplify the 164 bp fragment from EAEC mRNA cultured in LB and DMEM/tryptone for 2 h, and the expression of Pet mRNA increased in a time-dependent manner. Furthermore, EAEC delayed the transcription of Pet mRNA until after 8 h in MEM and DMEM without supplementation (Fig. 2b). Analysis of the kinetics of mRNA expression clearly showed the efficient expression of Pet mRNA induced by tryptone (Fig. 2c), evaluated as the mean of the area: intensity ratios measured by densitometry in three independent RT-PCR experiments. Interestingly, this upregulation was lost after 12 h of culture, indicating a delay in Pet mRNA expression in media without tryptone.

Pet-secreting EAEC damages HEp-2 cells infected in DMEM/tryptone

To investigate the damage to epithelial cells induced by Pet-secreting EAEC, we quantified adherent cells post-infection. HEp-2 cells were infected for 5 h with EAEC cultured in either DMEM or DMEM/tryptone, or infected with the isogenic mutant in DMEM/tryptone; cells without infection but in DMEM/tryptone were used as negative controls, constituting 100 % viable adherent cells. After infection, cells were fixed and stained with Giemsa. In HEp-2 cells infected with EAEC in DMEM without tryptone, cell damage was not detected (Fig. 3b) and the cells were similar to non-infected cells (Fig. 3a); the number of living cells among EAEC-infected cells in DMEM without tryptone was not significantly different from that without infection (Fig. 3e). However, damage was evident in cells infected with EAEC in DMEM/tryptone. Cell damage was characterized by cell rounding, picnotic nuclei, karyorrhexis, blebs in the cell membrane and cell detachment (Fig. 3c). The number of living cells after 5 h of infection with EAEC in DMEM/tryptone showed a dramatic decrease ($P < 0.001$) in comparison with the non-infected cells or infected cells in DMEM without tryptone (Fig. 3e).

To confirm that cell damage observed in epithelial cells infected with EAEC in DMEM/tryptone was induced by Pet, HEp-2 cells were infected with EAEC pet^- in DMEM/tryptone. Under this condition, no cell damage was observed (Fig. 3d). The mean count of viable HEp-2 cells infected with EAEC pet^- in DMEM/tryptone was significantly different from that of cells infected with wild-type EAEC in DMEM/tryptone, but not from that of cells infected with EAEC in DMEM without tryptone (Fig. 3e).

These results indicate that cell damage during EAEC infection depends on the secretion of Pet.

All these data indicate that EAEC causes cell damage in HEp-2 cells, using an *in vivo* model of infection in a culture medium that favours the secretion of Pet in DMEM supplemented with tryptone.

Pet internalization during epithelial cell infection with EAEC in DMEM/tryptone

To determine whether internalization of Pet into EAEC-infected cells was required for cell damage, as previously shown in cells incubated with purified Pet, HEp-2 cells were treated with purified Pet ($37 \mu\text{g ml}^{-1}$) for 3 h or infected with wild-type EAEC in DMEM/tryptone for 5 h. After infection, bacteria were killed with lysozyme, and HEp-2 cells were thoroughly washed and lysed to obtain the cytoplasmic fractions by cellular fractionation. The analysis of the cytoplasmic fraction from EAEC-infected cells by SDS-PAGE and Western blotting showed a 104 kDa protein band corresponding to Pet (Fig. 4, lane 2). Interestingly, this protein band was similar to that detected in the cytoplasmic fraction from cells treated with $37 \mu\text{g}$ purified toxin ml^{-1} (Fig. 4, lane 3).

Since Pet delivery by EAEC was efficient, we decided to confirm and quantify the internalization of Pet in a kinetics assay during EAEC infection by improving an intracellular immunofluorescent staining method for flow cytometry to detect Pet inside the cells. EAEC-infected HEp-2 cells were permeabilized and immunostained with anti-Pet antibodies and FITC-labelled secondary antibodies and the number of positive events (Pet inside the cells) was expressed by the shift of peak fluorescence intensity (FL_1). After 1 h of infection, a significant percentage (around 10 %) of Pet-FITC-positive cells was detected in comparison with non-infected cells (negative control), and the number of Pet-FITC-positive cells increased with infection time; after 5 h of infection, ~60 % of the cells were Pet-FITC-positive (Fig. 4b). These results indicate that Pet is efficiently internalized into the epithelial cells infected with EAEC cultured in DMEM/tryptone medium.

Since the efficient delivery of Pet from EAEC to the cells could be related to adhesion factors, we decided to compare the delivery of Pet from EAEC (aggregative phenotype) with that from an *E. coli* Pet hyperproducer (HB101pCEFN-1; minimal clone) in epithelial cells. A kinetics assay of HEp-2 cell infection (1–5 h) with EAEC, HB101 and HB101pCEFN-1 (*pet* minimal clone) was performed, and the internalization of Pet was quantified by intracellular immunofluorescence staining for flow cytometry. The internalization of Pet by EAEC was efficient from 1 h after infection, and the mean number of Pet-FITC-positive cells increased significantly in a time-dependent fashion (Fig. 4c). In contrast, in HEp-2 cells infected with HB101 or HB101pCEFN-1 (Pet hyperproducer), there were no Pet-FITC-positive cells at any

Fig. 3. Pet secreted during EAEC infection is able to damage epithelial cells grown in DMEM/tryptone. A cell adherence assay, for monitoring cell survival, was performed in HEp-2 cells infected with EAEC 042 using an m.o.i. of 10 from exponential-phase cultures. HEp-2 cells infected with EAEC 042 and cultured in DMEM (b) or DMEM/tryptone (c), and cells infected with EAEC 042pet⁻ and cultured in DMEM/tryptone (d). Uninfected cells cultured in DMEM/tryptone were used as controls (a). After 5 h of infection, cells were washed three times with PBS, fixed with 70 % methanol, stained with Giemsa and mounted in Permount resin. Magnification $\times 400$. (e) Number of living cells after infection. Adherent cells were quantified in five fields from six independent experiments. Data were statistically analysed by χ^2 test.

infection time (Fig. 4c). The shift of the fluorescence intensity peak of EAEC-infected cells after 5 h of infection was 62 % in comparison with those infected with HB101pCEFN-1 (Fig. 4c, insert). These results suggest that in addition to the secretion of Pet, a mechanism for efficient toxin delivery to the epithelial cells is required (i.e. bacterial adhesion).

Pet-producing EAEC decreases the live : dead cell ratio during infection

The efficient Pet internalization during EAEC infection must increase cell death induced by the toxin. To test this, HEp-2 cells were infected with wild-type EAEC, the isogenic *pet* mutant (EAECpet⁻) or the *pet*-complemented mutant (EAECpet⁻/pCEFN-1) in DMEM/tryptone medium. Infected cells were stained with Giemsa and analysed by light microscopy ($\times 40$). Additionally, an *in vivo* cell infection assay (unfixed infected cells) was performed in parallel and cells were stained with two vital stains, acridine

orange and ethidium bromide, and then analysed by flow cytometry. EAEC-infected cells showed a shift of the fluorescence intensity peak to form a second peak representing the cell-death population (Fig. 5b). This result correlated with a reduction in adherent cells detected by Giemsa staining (Fig. 5b) in comparison with non-infected cells (Fig. 5a). Statistical analysis from six experiments showed a significant reduction ($P < 0.001$) in the live : dead cell ratio after the infection with EAEC versus non-infected cells (Fig. 5e). These effects were not observed by light microscopy in HEp-2 cells infected with the isogenic mutant, since the cells were observed to look like non-infected cells, even though many bacteria were adhered in an aggregative pattern (Fig. 5c). These cells showed neither a shift of the fluorescence intensity peak nor changes in the cell-death population, and they were not significantly different from the non-infected cells (Fig. 5e). Furthermore, similar experiments using the *pet*-complemented strain (EAECpet⁻/pCEFN1) showed a shift of the fluorescence intensity peak, giving a second peak of dead

Fig. 4. Pet is efficiently internalized into epithelial cells during infection with EAEC 042. (a) Detection of Pet in the cytoplasmic fraction from EAEC-infected HEp-2 cells. Pet was identified by Western blotting (WB) in 50 μ g cytoplasmic proteins from cells infected for 5 h with EAEC in DMEM/tryptone (lane 2) or treated for 3.5 h with 37 μ g ml⁻¹ purified Pet (lane 3); as a control for detecting Pet, 0.3 μ g purified Pet protein was used (lane 1). (b) Kinetics of Pet detection in epithelial cells infected with EAEC. HEp-2 cells were cultured in DMEM/tryptone and infected with EAEC 042 in Petri dishes (60 mm) at various times (1–5 h) of infection. Cells were gently and thoroughly washed with PBS by centrifugation and permeabilized with PBS/0.05 % Triton X-100. Cells were immunostained using rabbit anti-Pet antibodies (1 : 20 000), followed by FITC-labelled goat anti-rabbit IgG antibodies (1 : 3000). Stained cells were analysed by flow cytometry (FL₁ shows the number of positive events). (c) Pet internalization depends on bacterial adhesion efficiency. HEp-2 cells were infected with EAEC 042, HB101, HB101(pCEFN-1) or HB101(pCEFN-2) at various times. Pet internalization was identified by flow cytometry and data were compared by the Kolmogorov–Smirnov test. Insert: cytometry analysis shows the peak fluorescence intensity shift due to EAEC-infected cells that were positive for Pet-FITC (63 %) in comparison with HB101(pCEFN-1)- or HB101(pCEFN-2)-infected cells.

Fig. 5. Pet-producing EAEC decreases the live : dead cell ratio during epithelial cell infection. HEp-2 cells cultured in DMEM/tryptone were infected with EAEC 042 (b) or the isogenic mutant EAEC 042pet⁻ (c), or the isogenic mutant complemented with the EAEC *pet* gene 042pet⁻(pCEFN-1) (d). HEp-2 cells without infection were used as controls (a). After 5 h of infection, cells were washed, fixed and stained with Giemsa. Live adherent cells were manually quantified. At the same time, an infection assay was performed *in vivo* (unfixed and non-permeabilized cells) (e). After the infection time, cells were washed and stained with acridine orange and ethidium bromide. Cells were analysed by flow cytometry to sort between live and dead cells. The histogram represents statistical analysis of six independent experiments, and the difference between treatments was analysed by χ^2 test.

cells; these results correlated well with cell morphological changes and the reduction in adherent cells detected by light microscopy (Fig. 5d). The number of cells decreased significantly ($P < 0.001$) in cultures infected with EAECpet⁻/pCEFN1 in comparison with uninfected cultures (Fig. 5e). Together, these data indicate that Pet secreted by EAEC during epithelial cell infection causes an increase in cell death.

Correlation among Pet secretion, internalization, cell detachment and death during infection of epithelial cells by EAEC

To determine the relationships among Pet secretion and internalization, cell detachment and death during HEp-2 cell infection by EAEC, we analysed all these events in six independent experiments. Pet secretion was detected by

Western blotting and densitometry in TCA-precipitated supernatants from EAEC-infected cells in DMEM/tryptone (Fig. 6, open squares). The internalization of Pet was quantified by intracellular immunofluorescent staining for flow cytometry (Fig. 6, filled squares). Cell detachment was quantified by Giemsa staining and light microscopy (Fig. 6, open triangles), whereas cell death was quantified by flow cytometry in cells stained with acridine orange and ethidium bromide (Fig. 6, filled triangles). The results, expressed as events per 100 (mean $\pm$ SD), showed that there was a direct correlation among all of these events. Thus, during infection of epithelial cells with EAEC, there was significant secretion of Pet after the first hour of infection. At this time, a significant increase in the internalization of Pet into the cells started, which was even more evident after 2 h. On the other hand, live cells decreased significantly after 3 h of infection. In the same way, the number of dead cells started to increase significantly after 3 h of infection. All of these events were progressive in relation to infection time (Fig. 6).

All these data indicate that epithelial cells infected with EAEC cultured in DMEM/tryptone for the secretion of Pet undergo morphological changes that lead to cell detachment and finally to cell death, and that bacterial adhesion, secretion of Pet, and internalization play important roles during EAEC infection.

DISCUSSION

Many Gram-negative pathogenic bacteria secrete proteins through the type V secretion system. These autotransporter proteins comprise a family of virulence factors (Henderson

et al., 2004; Loveless & Saier, 1997) characterized by a unique ability to promote their own secretion across the outer membrane of the bacterial envelope (Henderson *et al.*, 1998, 2004; Stathopoulos *et al.*, 2000). Those autotransporters from *Enterobacteriaceae* that possess a conserved serine protease motif (GDSGSP) in an analogous position within these proteins are classified into the SPATE protein family (Benjelloun-Touimi *et al.*, 1995; Brunder *et al.*, 1997; Eslava *et al.*, 1998; Guyer *et al.*, 2002; Henderson *et al.*, 1998, 1999a; Parham *et al.*, 2004; Patel *et al.*, 2004; Provence & Curtiss, 1994; Stein *et al.*, 1996). This serine protease motif is a key virulence factor in these proteins, since mutations of the motif or the use of specific serine protease inhibitors abolishes the proteolytic activity of these SPATEs (Henderson *et al.*, 2004). Thus, the catalytically active residue within this motif has been studied in different SPATEs by detecting the effect of these purified proteins on different substrates such as chromogenic peptides, purified proteins and cells, although this has not been analysed in an infection model that uses the SPATE-secreting bacteria directly on cell targets. In this study, we demonstrated that DMEM supplemented with tryptone allows efficient growth of EAEC and enables increased secretion of Pet by these bacteria. This increased secretion of Pet is transcriptionally favoured by the presence of tryptone. The amount of Pet secreted during epithelial cell infection by EAEC is enough to deliver an appropriate concentration of toxin inside the epithelial cells, and this causes a cytotoxic effect that leads to cell death.

Interestingly, Pet is not secreted by EAEC in DMEM, unlike the proteins secreted by the type III secretion systems (T3SSs) of other *E. coli* pathotypes, such as

Fig. 6. Loss of cell adherence and cell death of EAEC-infected epithelial cells correlates with the ability of EAEC to secrete and internalize Pet. HEp-2 cells were infected with EAEC 042 (m.o.i. 10) in DMEM/tryptone. Pet secretion was quantified from TCA-precipitated supernatant proteins by Western blotting and densitometry ($\square$). Pet internalization was quantified by flow cytometry detecting intracellular Pet-FITC-positive cells ($\blacksquare$). The cell adherence assay was performed with EAEC-infected cells, which were fixed and stained with Giemsa ($\triangle$). Each point represents the mean from six experiments measuring 300 cells per assay. Cell death analyses were also quantified by flow cytometry. HEp-2 cells were stained with acridine orange/ethidium bromide ($\blacktriangle$). Each point represents the mean of six experiments. For correlation purposes, the data are expressed as the percentage of events; error bars, SD.

enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (Kaper *et al.*, 2004). Moreover, EspC, an autotransporter homologous to Pet secreted by EPEC, is favoured in DMEM (Vidal & Navarro-Garcia, 2006); it is important to mention that EPEC T3SS and EspC are regulated by Ler (Elliott *et al.*, 2000). The combination of LB and DMEM allowed us to discover that tryptone is a factor that favours the secretion of Pet by EAEC. The secretion of Pet in DMEM/tryptone medium was related to transcriptional regulation instead of an accumulation of cytoplasmic Pet protein. These data suggest that gut luminal contents and composition are critical for secretion of Pet by EAEC in the intestinal epithelium. Similar phenomena have been observed in other pathogens. *Salmonella typhimurium* fails to infect mouse intestinal cells *in vitro* when inoculated in PBS, but invasion is enhanced with LB broth (Clark *et al.*, 1998). The authors speculate that the amino acid supply is an essential signal for cellular invasion, since addition of tryptone and yeast extract to PBS (two ingredients of LB broth) results in epithelial infection by *Salmonella*. Furthermore, maximum production of cholera toxin by El Tor strains of *Vibrio cholerae* requires AKI medium, which contains tryptone and bicarbonate (Iwanaga *et al.*, 1986), and increased amounts of flagellum production have been observed in EPEC and EHEC strains when they are grown on 1% tryptone (pH 7.2) agar plates (Erdem *et al.*, 2007). This tryptone-favoured secretion of Pet (where tryptone is a mixture of peptides formed by the digestion of casein by the protease trypsin) could be of relevance in exacerbating clinical manifestations in milk-drinking children infected with EAEC.

Since Pet is not secreted in DMEM media but is secreted in DMEM/tryptone, epithelial cell infection by EAEC in these different media was performed. These experiments clearly showed that Pet is an important virulence factor, since its absence during epithelial cell infection by EAEC (in DMEM but not in DMEM/tryptone) prevented the cytotoxic effects on HEp-2 and HT29 cells associated with purified Pet protein (Navarro-Garcia *et al.*, 1999). The lack of cytotoxic effects on the epithelial cells of EAEC in DMEM without tryptone was statistically similar to that observed with the *pet* isogenic mutant (EAEC pet^-). Previously, we found that human colonic tissue incubated with EAEC pet^- exhibited significantly fewer mucosal abnormalities both subjectively and morphometrically, as quantified by measurement of the crypt aperture diameter, than those caused by the wild-type EAEC (Henderson *et al.*, 1999b). Interestingly, the inhibition of Pet in DMEM appears to be specific, since Pic, another SPATE of EAEC, was still secreted (data not shown).

The pathogenesis of EAEC is complex, and EAEC strains are very heterogeneous (Elias *et al.*, 2002). Additionally, human and animal studies indicate that EAEC is able to bind to jejunal, ileal and colonic epithelium. No specific rearrangement of virulence factors has been associated with diarrhoea caused by EAEC. Our data clearly show that Pet

is involved in epithelial cell damage, as it is secreted and delivered into these cells to intoxicate them and cause cell death. These data also show that a very small dose of Pet is needed *in vivo* to cause the cytotoxic effects previously shown in an *in vitro* model using $37\ \mu\text{g ml}^{-1}$ purified Pet protein (Navarro-Garcia *et al.*, 1999, 2007b). Furthermore, to be internalized into epithelial cells, Pet has to be efficiently expressed, which appears to depend upon an appropriate environment, as mimicked by DMEM/tryptone medium versus DMEM alone. Once *pet* is expressed, the protein is secreted and also efficiently delivered to the epithelia, since the *in situ* EAEC-secreted Pet caused similar effects to those observed when $37\ \mu\text{g ml}^{-1}$ purified Pet protein was used. Moreover, efficient Pet delivery depends on other virulence factor(s), such as adhesion fimbriae, as a hyperproducer Pet minimal clone in *E. coli* HB101 was unable to cause effects on the epithelial cells, since this bacterium, unlike EAEC 042, was also unable to adhere to the epithelial cells. Interestingly, fimbriae in EAEC 042, in the strains that were unable to secrete Pet (by using DMEM without tryptone or an isogenic *pet* mutant), were capable of adhering to epithelial cells such as the Pet-secreting EAEC 042, although no cytotoxic effect was detected. These data suggest that these two factors are a complement to each other in forming part of the pathogenic effects of EAEC. Additionally, epidemiological studies have shown that strains hybridizing with at least one of the probes for AAF/II, Aap and Pet in Brazil, or with AAF/I, AAF/II, Pet, EAST and AspU in Nigeria, are more commonly recovered from children with diarrhoea than from healthy controls (Okeke *et al.*, 2000; Zamboni *et al.*, 2004). Interestingly, in many epidemiological studies, both Pet and AAF/II have been found at low prevalence (EAST1 and AAF/I are more prevalent) or to be absent (Boisen *et al.*, 2008; Vila *et al.*, 2000), although not in developing countries (Okeke *et al.*, 2000; Zamboni *et al.*, 2004). Furthermore, the *pet* gene is placed in between the two regions required for expression of the AAF fimbriae, as shown for the organization of biogenesis genes for AAF/II (Elias *et al.*, 1999). However, Pet and AAF/II are independently regulated, as AAF/II and the other adherence factors for EAEC are regulated by AggR. Our results were in accordance with this, since downregulation of the expression of Pet in MEM did not affect EAEC adherence. Moreover, the first report on the heterogeneity of EAEC showed that only the 042 strain, harbouring a combination of Pet and AAF/II, was able to cause diarrhoea in volunteers, but not strains 17-2, 34b or JM221 (Nataro *et al.*, 1995).

Thus, Pet itself is sufficient to cause the mucosal effects we have described, but an appropriate environment is needed for its expression as well as an efficient adherence factor (AAF/II) for its delivery to the epithelial cell. It is important to note that Pet appears not to be expressed in all the EAEC strains; however, Pet-positive strains might be of increased virulence, which might depend on the arrangement of genes (i.e. for AggR, AAF/II) for its efficient function.

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