***Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of ExoS**

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***Pseudomonas aeruginosa* is an opportunistic bacterial pathogen that primarily infects immunocompromised individuals and patients with cystic fibrosis. Using a tissue culture system, invasive strains of *P. aeruginosa* were discovered to induce apoptosis at high frequency in HeLa and other epithelial and fibroblast cell lines. This apoptotic phenotype in the infected cells was determined by several criteria including (i) visual changes in cell morphology, (ii) induction of chromatin condensation and nuclear marginalization, (iii) the presence of a high percentage of cells with subG1 DNA content, and (iv) activation of caspase-3 activity. Induction of the type III secretion machinery, but not invasion of *P. aeruginosa* is required for induction of apoptosis. The apoptosis phenotype is independent of the cytoskeletal rearrangements that occur in the host cell early after infection. Mutants in *P. aeruginosa* *exoS* fail to induce apoptosis and complementation with wild-type *exoS* restored the apoptosis-inducing capacity, demonstrating that ExoS is the effector molecule. Analysis of *exoS* activity mutants shows that the ADP-ribosylating capacity of ExoS is essential for inducing the apoptotic pathway.**

Keywords: ExoS, apoptosis, type III secretion, ADP-ribosylation

INTRODUCTION

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* causes devastating human infections in patients with cystic fibrosis, burns and/or immunosuppression (Tummler & Kiewitz, 1999; Salyers & Whitt, 1994). To successfully colonize and maintain an infectious cycle the organism carefully orchestrates production of a suite of virulence determinants including pili (Hahn, 1997; Wall & Kaiser, 1999) and non-pilus adhesins (Simpson *et al.*, 1995), extracellular enzymes (Ohman *et al.*, 1980; Salyers & Whitt, 1994), and exotoxins (Iglewski & Kabat, 1975). Some virulence factors are produced and secreted directly into the host cell using the cell-contact-mediated type III secretion machinery. To date, four such factors have been characterized, including ADP-ribosylating enzymes ExoS and ExoT (Frank, 1997; Frithz-Lindsten *et al.*, 1997; Yahr *et al.*, 1996a), an acute cytolytic factor ExoU (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998) and

an adenylate cyclase ExoY (Yahr *et al.*, 1998). Expression of these secreted effector molecules as well as components of the type III secretory apparatus are under the control of the transcriptional activator, ExsA (Hovey & Frank, 1995).

From a survey of various clinical isolates, Fleiszig *et al.* (1997) have divided isolates of *P. aeruginosa* into two categories, invasive and noninvasive strains, based on their abilities to invade mammalian cells. The phenotypes of these two classes are associated with the spectrum of virulence factors that are encoded within their genomes. Both types of strains harbour *exoT*. Typical invasive strains harbour *exoS* whereas noninvasive (i.e. cytolytic) strains harbour *exoU*. The cytolytic phenotype associated with noninvasive strains results mainly from the action of the *exoU* gene product (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). Although the factor(s) for the invasive phenotype is not clearly understood, Cowell *et al.* (2000) have recently shown that both ExoS and ExoT have an invasion-inhibitory effect on cytolytic *P. aeruginosa* strains. Despite extensive characterization of these factors including their abilities to cause morphological changes on various tissue culture cells (Finck-Barbancon *et al.*,

Abbreviations: FCS, foetal calf serum; p.i., post infection; PI, propidium iodide; TNF α , tumour necrosis factor α .

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
<i>Escherichia coli</i> strains		
DH5 α	F ⁻ ϕ 80 Δ lacZ Δ M15 <i>endA1 recA1</i> <i>hsdR17(r_k⁻m_k⁺) supE44 thi-1 relA1</i> Δ (lacZYA- <i>argF</i>)U169 <i>gyrA96 deoR</i>	Hanahan (1983)
SE5000	<i>araD139</i> Δ (<i>argF</i> -lac)U169 <i>rpsL150</i> (Str ^R) <i>relA1 fibB 5301 deoC1 ptsF25 rbsR recA56</i>	Gherardini <i>et al.</i> (1990)
<i>P. aeruginosa</i> strains		
PAK	Laboratory strain (invasive)	D. Bradley*
PAK <i>exsA</i> :: Ω	<i>exsA</i> knockout mutant derivative of PAK	Yahr <i>et al.</i> (1997)
PAK <i>exoS</i> :: Ω	<i>exoS</i> knockout mutant derivative of PAK	This study
PAK <i>exoT</i> :: <i>Gem</i>	<i>exoT</i> knockout mutant derivative of PAK	This study
PAK <i>exoS</i> :: Ω / <i>exoT</i> :: <i>Gem</i>	<i>exoS exoT</i> double mutant derivative of PAK	This study
388	Laboratory strain (invasive)	Iglewski <i>et al.</i> (1978)
388 <i>exoS</i> :: <i>Tc</i>	<i>exoS</i> knockout mutant derivative of 388	Kulich <i>et al.</i> (1995)
388 <i>exoT</i> :: <i>Tc</i>	<i>exoT</i> knockout mutant derivative of 388	Yahr <i>et al.</i> (1996a)
PAO1	Laboratory strain (invasive)	Holloway <i>et al.</i> (1979)
PA103	Laboratory strain (non-invasive and cytotoxic)	Liu (1966)
PA103 Δ <i>exoU</i> / <i>exoT</i> :: <i>Tc</i>	<i>exoU exoT</i> mutant derivative of PA103	Vallis <i>et al.</i> (1999)
Plasmids		
pDN18	Broad-host-range plasmid, IncP, Tc ^r	Nunn <i>et al.</i> (1990)
pDN19	Broad-host-range plasmid, IncP, Tc ^r	Nunn <i>et al.</i> (1990)
pPC110	Gentamicin cassette in pUC7, Ap ^r Gen ^r	Nunn <i>et al.</i> (1990)
pUC19 Ω	pUC19 with Ω insertion	Totten & Lory (1990)
pHW9945	<i>exoS</i> from PAK cloned into pCR2.1-TOPO	This study
pHW9950	<i>exoS</i> in pHW9945 disrupted by an Ω insertion	This study
pHW9946	<i>exoT</i> from PAK cloned into pCR2.1-TOPO	This study
pHW9951	<i>exoT</i> in pHW9946 disrupted by a gentamicin-resistance cassette	This study
pHW9948	<i>exoS</i> of PAK cloned in pDN19, Tc ^r	This study
pHW9949	<i>exoT</i> of PAK cloned in pDN18, Tc ^r	This study
pUCP18	Broad-host-range cloning vector, IncP, Ap ^r	Vallis <i>et al.</i> (1999)
pUCPG18	pUCP18 with gentamicin selection marker, Ap ^r Gen ^r	This study
pUCP <i>exoS</i>	<i>exoS</i> from strain 388 cloned into pUCP18, Ap ^r	Vallis <i>et al.</i> (1999)
pUCPG <i>exoS</i>	pUCP <i>exoS</i> with gentamicin selection marker, Ap ^r Gen ^r	This study
pUCP <i>exoSE381A</i>	<i>exoS</i> of pUCP <i>exoS</i> mutated into E381A, Ap ^r	Vallis <i>et al.</i> (1999)
pUCPG <i>exoSE381A</i>	pUCP <i>exoSE381A</i> with gentamicin selection marker, Ap ^r Gen ^r	This study
pUCP <i>exoT</i>	<i>exoT</i> from strain 388 cloned into of pUCP18, Ap ^r	Vallis <i>et al.</i> (1999)
pUCPG <i>exoT</i>	pUCP <i>exoT</i> with gentamicin selection marker, Ap ^r , Gen ^r	This study

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1997; Hauser *et al.*, 1998; Yahr *et al.*, 1998; McGuffie *et al.* 1999; Vallis *et al.*, 1999), the mechanisms by which these virulence factors alter mammalian cell physiology remain enigmatic.

While investigating bacterial genes that are stringently induced during infection of tissue culture cells, we noted that invasive strains of *P. aeruginosa* produce cell-contact-dependent factors that induce an apoptosis-like

morphology in cultured cells. Further characterization of this phenotype demonstrated that the ADP-ribosylating activity of ExoS is required for induction of programmed cell death by invasive *P. aeruginosa*.

METHODS

Bacterial strains and plasmids. Bacterial cultures were grown in Luria-Bertani broth (L-broth) at 37 °C. Strains and plasmids used in this study are listed in Table 1. The type III regulatory mutant *PAKexsA::Ω* (Yahr *et al.*, 1997), and the *exoS* and *exoT* mutant strains of 388 have been described previously (Kulich *et al.*, 1995; Yahr *et al.*, 1996b). The *exoS*, *exoSE381A* and *exoT* clones of strain 388 were derived from pUCPexoS, pUCPexoSE381A and pUCPexoT, respectively (Vallis *et al.*, 1999), by inserting a 1.6 kb *EcoRI* fragment containing the gentamicin-resistance cassette from pPC110 (Nunn *et al.*, 1990) into *HindIII* sites, resulting in pUCPGexoS, pUCPGexoSE381A and pUCPGexoT, respectively. The corresponding vector control pUCPG18 was generated by inserting the gentamicin cassette into the *EcoRI* site of pUCP18.

The *exoS* and *exoT* genes were PCR amplified from the PAK chromosome using the following two pairs of primers: *exoS*-1, 5'-CAG TTG TTC GAG TTG ATG GTG GAT CTG GGC CCT GT-3' and *exoS*-2, 5'-CGT TTC GTC GCC TGG ACC TAC CTC GAC AAG AAG CA -3', and *exoT*-1 5'-AGG AAG GTC ATC AGC AGG GCG ATC TCG GTG GTC AT-3' and *exoT*-2 5'-GCT GTA CGG CGC AAA TGA AAA CGG ACA CCC CTT GG-3', respectively. Cloned *exoS* and *exoT* genes in pCR2.1-TOPO (pHW9945 and pHW9946) were disrupted by insertion of the Ω cassette (resistant to streptomycin and spectinomycin) or gentamicin-resistance cassette, respectively, to generate pHW9950 and pHW9951. Linearized plasmid DNA containing the disrupted genes were double crossed into the chromosome of PAK by electroporation to generate *PAKexoS::Ω*, *PAKexoT::Gem* and *PAKexoS::Ω/exoT::Gem* mutant strains. The mutant strains were confirmed by Southern hybridization as well as by defects in ExoS and/or ExoT secretion into the medium under low-calcium type III inducing conditions (Yahr *et al.*, 1997). Briefly, overnight bacterial cultures in L-broth containing 5 mM EGTA were diluted 10-fold into fresh L-broth containing 5 mM EGTA. The cultures were shaken at 37 °C for 6 h and culture supernatants were recovered by centrifugation. Culture supernatants (0.5 ml) were concentrated down to 10 µl using Centricon-50, mixed with an equal volume 2 × loading buffer, boiled and subjected to 10% SDS-PAGE analysis. Protein bands were visualized after staining with Coomassie blue.

Cells and media. HeLa S3 cell line in suspension culture was maintained in Joklik's modified minimal essential medium supplemented with 100 µg penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ and 7.5% horse serum (MEM+7% HS; Gibco). For the bacterial infections, HeLa monolayers were plated from suspension culture on the previous day. HeLa cell monolayers were maintained in Dulbecco's modified medium supplemented with 5% foetal calf serum (DMEM+5% FCS; Gibco). All other cell lines were maintained as monolayers in DMEM+10% FCS.

Cell infection by *Pseudomonas*. Overnight bacterial cultures were pelleted and resuspended in DMEM. Cell monolayers were grown to >80% confluency in 6-well plates (~5 × 10⁵ cells per 35 mm well) and washed with PBS. The cell

monolayer was inoculated with 1 × 10⁷ bacteria resuspended in 1 ml DMEM (m.o.i. = 20) and incubated for 2 h at 37 °C in a 5% CO₂ incubator. The bacterial inoculum was removed, cells were washed with PBS and fresh DMEM+5% FCS supplemented with 200–400 µg gentamicin ml⁻¹ was added to the monolayer. Cells were incubated in 5% CO₂ at 37 °C for an additional 3–24 h. As positive controls for apoptosis, HeLa cells were incubated with 10 ng tumour necrosis factor α (TNFα) ml⁻¹ plus 20 µg cycloheximide ml⁻¹.

Bacterial invasion tests. Cell monolayers were infected by the bacteria for 2 h and washed once with phosphate buffered saline. Subsequently, cells were grown for an additional 2 h in fresh DMEM+5% FCS supplemented with 400 µg gentamicin ml⁻¹ or 400 µg amikacin ml⁻¹ to kill extracellular organisms. Cells were harvested by scraping and lysed with 0.1% Triton X-100 prior to serial dilution and plating on L-agar plates with appropriate antibiotics to obtain colony counts. Tests were performed in triplicate and the mean of three trials is reported. To inhibit invasion, the cell monolayers were incubated with the cytoskeletal poison cytochalasin D (10 µM) or the tyrosine kinase inhibitor genestein (200 µM) for 2 h prior to the addition of bacteria.

Apoptosis assays.

Caspase-3 assay. Caspase-3 activities were measured using a commercially available kit (Caspase-3 cellular activity assay kit plus; BioMol). HeLa cells (3 × 10⁷) were infected with *P. aeruginosa*, then cells were washed and harvested by scraping and centrifugation (1000 g, 10 min) at various times post-infection (p.i.). HeLa cells were lysed with 0.1% Tween 20 and cell lysates (supernatants) were saved following centrifugation at 10000 g for 10 min. Dilutions of the cell lysates in a 96-well plate were incubated with the DEVD-pNA substrate, or the substrate plus caspase-3 inhibitor (supplied with the assay kit). Changes in OD₄₀₅ were followed for 2 h at 10 min intervals. Protein concentration was determined using the Bio-Rad Protein Assay System. The specific activity is reported as pmol substrate cleaved min⁻¹ (µg protein)⁻¹.

Hoechst staining of condensed chromatin. Infected cells were recovered at 20 h p.i. by trypsinization of the cell monolayer. Cells were washed once with PBS and stained with Hoechst 33258 (1 mg ml⁻¹) for 10 min in the dark. Chromatin condensation was examined under the fluorescence microscope using a DAPI filter.

Flow cytometry analyses of subG1 DNA populations. Bacterial infected cells were harvested at 24 h p.i. by trypsinization of the cell monolayers and fixation overnight at 4 °C in 70% ethanol. Cells were harvested by centrifugation and stained for 30 min in 500 µl propidium iodide (PI) staining solution (0.1% BSA, 0.1% RNase, 0.5 mg PI ml⁻¹). Cells were directly counted in batches of 10000 and shown by plotting PI fluorescence versus cell number. The percentage of apoptotic cells was calculated using the internal software system of the flow cytometer.

RESULTS

P. aeruginosa PAK induces apoptosis in cell cultures

PAK is an invasive strain of *P. aeruginosa* (Fleiszig *et al.*, 1997). Infection of HeLa S3 cells by this strain was studied as an *in vitro* model system of bacterial–host interactions. Cultured cell monolayers were infected with wild-type PAK and subsequently followed to

Fig. 1. Morphology and Hoescht staining of HeLa cell line S3 infected with *P. aeruginosa*. HeLa cells were incubated with no bacteria (a, b and c), PAK (d, e and f), PA103 (g, h and i), or TNF α plus cycloheximide (j, k and l). Images in the first column (a, d, g and j) were captured at 3 h p.i., whereas remaining (second and third columns) images were at 24 h p.i. with 10-fold higher magnification than the first column. Cells were stained with Hoescht 33258 and viewed under fluorescent light (b, e, h and k) or identical view under regular light (c, f, i and l) are shown. Panels (a), (d), (g) and (j) were viewed under natural light with no staining.

characterize any infection-specific morphological changes. By 3 h p.i., the HeLa cells adopted a rounded morphology and subsequently detached from the monolayer (Fig. 1d). By 24 h p.i., the cells displayed a typical apoptotic morphology as determined by membrane blebbing, cell shrinkage and the presence of apoptotic bodies (Fig. 1f) as compared to uninfected controls (Fig. 1a, c).

To confirm that this morphological alteration was due to programmed cell death, a number of apoptosis-specific assays were performed. Caspase-3 is an apoptosis-specific protease and various apoptotic pathways lead to the proteolytic activation of this protease from its proenzyme form (Kothakota *et al.*, 1997). Following infection with PAK, caspase-3 activity is detected in HeLa cell lysates within 5 h p.i. (Fig. 2) and this activity is blocked by the caspase-3-specific inhibitor DEVD (N-acetyl-Asp-Glu-Val-Asp; BioMol). Fragmentation of DNA, another indicator of the late events of apoptosis, results in the appearance of cells containing subG1 levels of DNA that can be detected by flow cytometry analyses of PI-stained cells (Fraker *et al.*, 1995). By 24 h p.i., 29% of cells infected with strain PAK are hypodiploid in DNA content (Fig. 3b) whereas subG1 levels of DNA are found in only 5% of the uninfected control HeLa S3 cells (Fig. 3a). The correlation of apoptosis induction with an increased subG1 cell population is also seen after cells are incubated in the presence of TNF α and cycloheximide, conditions known to induce apoptosis (Fig. 3d). Interestingly, the subG1 population of PAK-infected cells seemed to be

Fig. 2. Induction of caspase-3 activity following infection with *P. aeruginosa*. HeLa cells were infected with either the wild-type PAK (■) or a type III mutant strain PAKexsA:: Ω (▲). Untreated (●) and TNF α /cycloheximide (x) treated cells were used as negative and positive controls, respectively. The means of three independent tests are shown.

Fig. 3. Histogram of cell number versus PI uptake to demonstrate DNA fragmentation in HeLa cells. HeLa cells were subjected to the following treatments: (a) uninfected control, (b) infection with wild-type PAK, (c) infection with type III defective mutant PAKexsA::Ω and (d) treated with TNFα/cycloheximide. Intensity of fluorescence (FL1-A) was plotted against the number of HeLa cells.

Table 2. Induction of apoptosis by various bacterial strains

ND, Not done; CytoD, cytochalasin D; + and – in the table body represent detectable and undetectable changes, respectively.

Strain	Invasion (%) [*]		Apoptotic morphology (24 h p.i.)		Caspase-3 activity (5 h p.i.)	PI uptake (% apoptosis) [†]
	− CytoD	+ CytoD				
	− CytoD	+ CytoD				
PAK	2.6	0.009	+	+	+	33
PAO1	0.6	0.004	+	+	+	ND
388	ND	ND	+	+	+	14
PA103	0	0	−	−	−	4
PAK <i>exsA</i> ::Ω	2.4	0.004	−	−	−	5

* Mean values of three trials.

† Percentage apoptosis was based on subG1 population by FACS analysis.

derived from G1-phase cells, as the appearance of subG1 cells correlated with the loss of an equivalent portion of the G1 population whereas the S+G2 phase cell population remained unchanged (Figs 3 and 6, and data not shown), suggesting that the apoptosis was induced selectively during infection of cells in phase G1 of the cell cycle. In addition, chromatin condensation and marginalization of nuclei was apparent in PAK-infected cells as assessed by staining with the DNA dye Hoechst 33258 (Fig. 1e). These nuclear changes are diagnostic for apoptosis and were consistent with the flow cytometry

analyses. Thus, by several criteria, PAK infection rapidly induces apoptosis.

To determine whether PAK-induced apoptosis was a general phenomenon, additional cell lines derived from different lineages were examined. These included canine kidney epithelial cells (MDCK), primary mouse fibroblasts, a human melanoma cell line (MEWO) and breast cancer cell lines (MCF7, HBL100 and MDAMB231), monkey kidney cells (Vero), and a human lung epithelial cell line (A549). PAK-induced morphological changes

similar to those observed in HeLa S3 cells were seen in MEWO, Vero, mouse fibroblast, MDCK and breast cancer cell lines but not in A549 (data not shown). These results indicate that transformed tumour cell lines as well as non-transformed cell lines undergo apoptosis upon infection by the PAK strain; thus the apoptosis response by mammalian cells upon PAK infection is not unique to the HeLa cell line.

Invasive strains of *P. aeruginosa* are more potent than the cytolytic strain PA103 at triggering apoptosis

It was noticed in the initial studies that in addition to PAK, the prototypical invasive strain, two other invasive strains (PAO1 and 388) induced consistently high levels of apoptosis. In contrast, in cells infected by the noninvasive, cytolytic strain PA103, apoptotic responses were inapparent or below the detection levels of the assays (Table 2). Although morphological changes were observed within 3 h p.i., the PA103-infected HeLa cells displayed a classic necrotic morphology, including cell swelling and subsequent lysis (Fig. 1g–i). Furthermore, flow cytometry analysis of PI-stained PA103-infected cells for subG1 DNA levels showed only background levels of DNA fragmentation. Finally, caspase-3 activity, a definitive marker for apoptosis, was absent in PA103-infected cells (Table 2). This suggested that apoptosis induction appeared to correlate with the invasive phenotype of the strain.

Induction of apoptosis is independent of bacterial invasion but dependent on the type III secretion machinery

Because apoptosis induction appeared to correlate with the invasive phenotype of the strain, it was important to determine whether the apoptotic pathway was dependent on bacterial invasion. Thus, gentamicin-exclusion assays were conducted in the presence of the cytoskeletal inhibitor cytochalasin D (Verschuere *et al.*, 1995). As shown in Table 2, cytochalasin D blocked bacterial invasion completely, yet induction of apoptosis continued to occur. Similar results were obtained with genistein (Evans *et al.*, 1998), another inhibitor of bacterial invasion (data not shown). Consistent with its noninvasive phenotype, the cytotoxic strain PA103 could not be recovered from HeLa cells in the invasion assay. Background invasion was determined by infection with the non-invasive *E. coli* strain SE5000 (Gherardini *et al.*, 1990), which invades at a frequency of 0.0008% in the absence of cytochalasin D or genistein. Thus, induction of apoptosis is independent of invasion.

Although bacterial invasion was not required for apoptosis induction, contact between bacteria and eukaryotic cells, and subsequent expression of the contact-dependent type III secretion system might still be required. ExsA is a global regulator of the type III secretion machinery (Hovey & Frank, 1995). Examination of monolayer infections with PAK or an isogenic *exsA*

Fig. 4. Secretion of ExoS and ExoT under type III inducing conditions. Secreted proteins in type III inducing medium were separated by 10% SDS-PAGE and visualized by staining with Coomassie blue. Lane 1, wild-type PAK; lane 2, PAKexoS::Ω with vector pDN19; lane 3, PAKexoS::Ω complemented with wild-type *exoS* clone, pHW9948; lane 4, PAKexoT::Gem with vector pDN18; lane 5, PAKexoT::Gem complemented with *exoT* clone, pHW9949; lane 6, PAKexoS::Ω/*exoT* double mutant with vector pDN19; lane 7, PAKexoS::Ω/*exoT* double mutant complemented with *exoS* clone pHW9948; lane 8, PAKexoS::Ω/*exoT* double mutant complemented with *exoT* clone pHW9949. M, protein standard marker.

mutant revealed a profound defect in the ability of the type III mutant to provoke apoptosis. Although the invasion frequency for the *exsA* strain is nearly identical to that of wild-type PAK (Table 2), infection with the *exsA*-deficient strain fails to induce caspase-3 activity (Fig. 2) and produces a level of DNA fragmentation that is not significantly different from uninfected controls (Fig. 3c). No morphological changes, specifically cell rounding and lifting, were observed in monolayers infected with the *exsA* mutant even after 24 h. These results demonstrate that the expression of the apoptosis-inducing factor is under the control of ExsA, suggesting a type III dependent secreted protein.

ExoS is the apoptotic inducer

Among the four type III dependent effector molecules known, only *exoT* is found in both invasive and non-invasive strains of *P. aeruginosa*, whereas *exoS* and *exoY* are specifically encoded by the invasive strains (PAK, PAO1 and 388) and *exoU* by the cytolytic strains (PA103) (Fleiszig *et al.*, 1997; Yahr *et al.*, 1998). Although all four Exo products were shown to affect host-cell morphology when expressed independently (Vallis *et al.*, 1999), alterations in host cell morphology appear to be predominantly due to the action of ExoS and ExoT (McGuffie *et al.*, 1999; Olson *et al.*, 1999). To test the possible involvement of ExoS and ExoT in the HeLa cell apoptosis, isogenic mutants of *exoS*, *exoT* and an *exoS exoT* double mutant were generated in the PAK background. To confirm that these strains were ExoS and/or ExoT deficient, gene expression in these mutant strains was induced under type III secretion conditions (Yahr *et al.*, 1997). As expected, the mutant strains failed to secrete the corresponding ExoS and/or ExoT proteins as determined by SDS-PAGE (Fig. 4). Apoptotic in-

Fig. 5. Induction of caspase-3 activity following infection with *P. aeruginosa* strains. HeLa cells were infected with *P. aeruginosa*; cell lysates were made at 5 h p.i. and assayed for caspase-3 activity. Assays were done in triplicate and means are shown. (a) Results from cells infected with PAK derivatives. CTRL, uninfected control; PAK, wild-type strain; ExsA, PAKexoA::Ω; ExoS, PAKexoS::Ω; ExoS/V, PAKexoS::Ω(pDN19); ExoS/S, PAKexoS::Ω(pHW9948); ExoT, PAKexoT::Gem; ExoST, PAKexoS::Ω/exoT::Gem; ExoST/V, PAKexoS::Ω/exoT::Gem(pDN19); ExoST/S, PAKexoS::Ω/exoT::Gem(pHW9948); ExoST/T, PAKexoS::Ω/exoT::Gem(pHW9949). (b) Results from cells infected with strain 388 derivatives. CTRL, uninfected control; PAK, wild-type strain; 388, wild-type strain; ExoS, 388exoS::Tc; ExoT, 388exoT::Tc; ExoS/V, 388exoS::Tc(pUCPG18); ExoS/S, 388exoS::Tc(pUCPGexoS); ExoS/SM, 388exoS::Tc(pUCPGexoS381A).

duction was assessed in cells infected with these mutants. Consistent with previous studies with similar mutants in strain 388, both PAKexoS and PAKexoT mutants failed to induce the early cytoskeletal rearrangements that manifest as cell rounding and detachment after infection. However, the PAKexoS/exoT double mutant failed to cause cytoskeletal rearrangement even after 24 h incubation. Apoptosis in *exoT* mutant infected cells as determined by caspase-3 activity, DNA fragmentation and chromatin condensation continued unabated at wild-type levels (Fig. 5 and data not shown). These data indicate that ExoT is not an inducer of apoptosis. In contrast, apoptosis was completely abolished in PAKexoS- and PAKexoS/exoT-infected cells. This defect was complemented back to wild-type levels by introduction of an *exoS* gene clone (pHW9948) but not an *exoT* gene clone (pHW9949), demonstrating that ExoS is the apoptotic inducer. This was further confirmed by using *exoS* and *exoT* mutations in the 388 background. Again, 388exoS::Tc was non-apoptotic while 388exoT::Tc was as potent as wild-type in induction of apoptosis (Fig. 5). The apoptotic potential of the *exoS* mutant was completely complemented back to the wild-type level by introducing a plasmid containing *exoS* from 388, pUCPGexoS.

ADP-ribosylating activity of ExoS is essential for apoptosis induction

Structure–function studies of ExoS have previously shown that this 49 kD protein possesses two distinct functional domains. The N-terminal domain is capable

of disrupting the actin cytoskeleton and causing cell rounding through interaction with the host Rho factor, while its C-terminal domain has an ADP-ribosylating activity that is capable of modifying the eukaryotic Ras protein (Pederson *et al.*, 1999). A mutant form of ExoS, ExoSE381A, has a glutamic acid residue changed into alanine at position 381, eliminating the ADP-ribosylase activity (Liu *et al.*, 1996). This mutant form of *exoS* failed to complement the 388exoS mutant for the apoptosis induction (Fig. 5b). In contrast, this mutant caused significant levels of cell rounding and lifting, indicating effective delivery of the protein. Indeed, this protein is secreted to high levels under type III inducing conditions (data not shown). These data suggest that ADP-ribosylation of a cellular protein by ExoS is required to trigger apoptosis. Importantly, these data also indicate that the changes in cell morphology are likely to be independent of the apoptosis and mediated through a different cellular pathway.

These studies thus far indicate that ExoS is necessary for apoptosis induction but it may not be sufficient. Thus, to determine whether other exotoxins produced by invasive strains might also be required, further complementation studies were performed in the background of PA103. To remove the cytolytic phenotype, a PA103 strain deficient in both ExoU and ExoT production was used. The strain, PA103Δ*exoU/exoT*::Tc, is both non-cytolytic as described earlier (Vallis *et al.*, 1999) and an apoptotic null strain (Fig. 6). Introduction of a wild-type *exoS* gene from strain 388, pUCPGexoS, resulted in a strain capable of triggering apoptosis to high levels,

Fig. 6. Flow cytometry analysis following PI staining of HeLa cells infected with (a) PA103/ Δ exoU/exoT::Tc(pUCP18); (b) PA103/ Δ exoU/exoT::Tc(pUCPexoS); (c) PA103/ Δ exoU/exoT::Tc(pUCPexoSE381A); and (d) PA103/ Δ exoU/exoT::Tc(pUCPexoT). The intensity of fluorescence (FL1-A) was plotted against the number of HeLa cells.

while introduction of the mutant *exoSE381A* gene, pUCPGexoSE381A, failed to rescue the apoptosis-inducing activity (Fig. 6). These confirm the requirement for the ExoS-mediated ADP-ribosylating activity in triggering apoptosis.

DISCUSSION

The *P. aeruginosa* ADP-ribosylating toxins ExoS and ExoT play a crucial role in bacterial dissemination to the bloodstream (Frank, 1997). These toxins have also been implicated in alterations of cytoskeletal structure, particularly actin, and signalling via the Ras-dependent pathway in eukaryotic hosts (Frithz-Lindsten *et al.*, 1997). ExoS and ExoT of invasive strains share 75% amino acid identity, yet ExoT possesses only 0.2% of the ADP-ribosylating activity of ExoS. A recent report (Hauser & Engel, 1999) has suggested that *P. aeruginosa* strain PA103 causes a type III secretion dependent apoptosis in macrophages and epithelial cells by a mechanism independent of ExoU. In that study, three criteria were used to evaluate apoptosis: terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling assays, cytoplasmic nucleosome assays and Hoechst staining. Under our assay conditions, PA103 infection failed to induce significant levels of either subG1-like cells or apoptosis-specific caspase-3 activity. Furthermore, an *exoU* *exoT* double mutant derivative of PA103 is nonapoptotic, and introduction of an *exoS* clone into the mutant strain clearly restored the ability to cause HeLa cell apoptosis whereas introduction of an *exoT* clone resulted in no apoptosis-inducing ability (Fig. 6). However, our data

do not rule out the possibility of low-level apoptosis caused by PA103, especially considering the fact that PA103 secretes ExoT, which is highly homologous to ExoS and also has a low ADP-ribosylating activity. It is possible that our assay systems may not be sensitive enough to detect a low level of apoptosis caused by the cytolytic strain PA103.

Previous studies had suggested that apoptosis might be induced by other *P. aeruginosa* gene products. Introduction of purified exotoxin A or porin proteins into mammalian cells has previously been shown to cause apoptosis (Buommino *et al.*, 1999; Hafkemeyer *et al.*, 1999; Morimoto & Bonavida, 1992). However, the ability of these molecules to induce apoptosis within the context of bacterial infection remains unproven. In addition, it has previously been shown that exotoxin A played no role in the tissue culture and acute infection models (Apodaca *et al.*, 1995). This is consistent with our observation that the *exoA* mutant strain of PAK is unaltered in its ability to cause apoptosis of HeLa cells (data not shown) and the PA103 strain, which constitutively expresses high level of exotoxin A (Liu, 1966), failed to cause apoptosis.

YopE of *Yersinia* and SptP of *Salmonella* are homologues of *P. aeruginosa* ExoS and are also secreted by type III machinery. The homology is limited to the GTPase-activating-protein (GAP) binding domain, which mediates actin disruption, and is not found in the ADP-ribosylating domain (Frithz-Lindsten *et al.*, 1997; Pederson *et al.*, 1999). In addition, SptP has a tyrosine phosphatase domain at the C terminus while YopE has none. Neither YopE nor SptP have been

reported to have the apoptotic-inducing phenotype. This is consistent with our observation that it is the ADP-ribosylating activity unique to ExoS and not the common GAP-binding domain that is responsible for the induction of apoptosis.

A number of other bacterial pathogens are known to cause host cell apoptosis by type III secreted effector molecules. For example, IpaB of *Shigella* spp. and SipB of *Salmonella* spp. trigger apoptosis by directly activating caspase-1 (Hilbi *et al.*, 1999; Hersh *et al.*, 1999) while YopJ/P of *Yersinia* spp. cause apoptosis by inhibiting the NF- κ B-mediated pathway (Orth *et al.*, 1999). Similar to these examples, our data suggest *P. aeruginosa* also induces apoptosis by one of its type III secreted proteins. ExoS possesses two functional domains with its N-terminal 234 amino acids capable of causing cell rounding and actin rearrangement in a Rho-dependent manner whereas the C-terminal 221 residues have a FAS-dependent ADP-ribosyltransferase activity (Pederson *et al.*, 1999). In addition, it has been demonstrated that exposure to ExoS-producing bacteria, but not non-producing mutants, caused cultured mammalian cells to round up and prevented entry into S phase (McGuffie *et al.*, 1998). Consistent with this observation, flow cytometry analysis of PI-stained infected cells indicates that *P. aeruginosa* preferentially induced apoptosis in G1-phase cells. It remains to be determined whether the apoptotic phenotype and the ability of ExoS to block the G1/S transition are related.

Caspase-3 activity is detected in cell lysates as early as 5 h p.i. with PAK. *In vivo* expression of the type III secretion system is induced and ExoS synthesis is induced only after bacteria–host cell contact. Thus, the interval during which the host cell becomes committed to apoptosis as a result of ExoS function is probably significantly shorter than the 5 h seen here. This rapidity suggests that ExoS may be acting directly to activate apoptotic pathways in the cell. *In vitro*, ExoS ADP-ribosylates several host proteins, including intracellular proteins such as the intermediate filament protein vimentin (Coburn *et al.*, 1989a), several low-molecular-mass GTP-binding proteins (Coburn *et al.*, 1989b) and extracellular proteins such as human immunoglobulin 3 and apolipoprotein A1 (Knight & Barbieri, 1997). *In vivo*, ExoS has been shown to specifically ADP-ribosylate Ras proteins, uncoupling Ras-mediated signal-transduction pathways (Vincent *et al.*, 1999; McGuffie *et al.*, 1998; Ganesan *et al.*, 1998, 1999). Among the four Ras proteins (H-Ras, N-Ras, K-RasA and K-RasB), H-Ras was found to be ADP-ribosylated most extensively. Ras is a molecular switch which controls cellular processes in response to extracellular stimuli. GTP-bound forms of Ras can activate downstream effectors (including Raf-1) which initiate MAP kinase pathways that regulate gene expression affecting cellular proliferation, differentiation and apoptosis. Of interest are also the GTPase-activating proteins (GAP), such as Ras-GAP, which accelerate the intrinsic GTPase activity of Ras resulting in inactive GDP-bound forms of Ras (McNeill & Downward, 1999). Thus, activation of

MAP kinase pathways through ADP-ribosylation of Ras is an attractive mechanistic model for the ExoS-mediated apoptosis. However, the exact pathway(s) by which ExoS triggers apoptosis remains to be elucidated and may involve other yet-to-be-identified cellular targets of the ExoS ADP-ribosyltransferase.

ACKNOWLEDGEMENTS

We thank Dara Frank for providing strains and making suggestions, K. Gilbert and the Flow Cytometry Resource Center at the University of Arkansas for Medical Sciences for technical assistance and helpful discussions, and Peter Turner for advice on the caspase-3 assays. R. Kurten kindly provided resources for fluorescence microscopy. M.R.K. is supported by the Honour's Research Program of the University of Arkansas College of Medicine. This work was supported by NIH AI39524 (to S.J.), AI42390 and AI22627 (to M.C.).

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Received 28 February 2000; revised 17 May 2000; accepted 25 May 2000.