Secreted products of a nonmucoid *Pseudomonas aeruginosa* strain induce two modes of macrophage killing: external-ATP-dependent, P2Z-receptor-mediated necrosis and ATP-independent, caspase-mediated apoptosis

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A nonmucoid clinical isolate of *Pseudomonas aeruginosa*, strain 808, elaborated ATP-dependent and ATP-independent types of cytotoxic factors in the growth medium. These cytotoxic factors, active against macrophages, were secreted during the exponential phase of growth in a complex medium. Commensurate with the appearance of the cytotoxic activities in the cell-free growth medium, several ATP-utilizing enzymic activities, such as adenylate kinase, nucleoside diphosphate kinase and 5'-nucleotidase (ATPase and/or phosphatase), were detected in the medium. These ATP-utilizing enzymes are believed to convert external ATP, presumably effluxed from macrophages, to various adenine nucleotides, which then activate purinergic receptors such as P2Z, leading to enhanced macrophage cell death. Pretreatment of macrophages with periodate-oxidized ATP (oATP), which is an irreversible inhibitor of P2Z receptor activation, prevented subsequent ATP-induced macrophage cell death. A second type of cytotoxic factor(s) operated in an ATP-independent manner such that it triggered activation of apoptotic processes in macrophages, leading to proteolytic conversion of procaspase-3 to active caspase-3. This cytotoxic factor(s) did not appear to act on procaspase-3 present in macrophage cytosolic extracts. Intact macrophages, when exposed to the cytotoxic factor(s) for 6–16 h, underwent apoptosis and demonstrated the presence of active caspase-3 in their cytosolic extracts. Interestingly, two redox proteins, azurin and cytochrome *c*₅₅₁, were detected in the cytotoxic preparation. When cell-line-derived or peritoneal macrophages or mast cells were incubated overnight with Q-Sepharose column flow-through fraction or with a mixture of azurin and cytochrome *c*₅₅₁, they underwent extensive cell death due to induction of apoptosis.

Keywords: ATP-utilizing enzymes, *Pseudomonas aeruginosa* virulence, programmed cell death, azurin, cytochrome *c*₅₅₁

INTRODUCTION

Introduction of cytotoxic factors into phagocytic cell

Abbreviations: Ak, adenylate kinase; CF, cystic fibrosis; LDH, lactate dehydrogenase; Ndk, nucleoside diphosphate kinase; oATP, oxidized ATP.

cytosol to cause cell death is a major weapon in the arsenal of many pathogens. Thus *Shigella* and *Salmonella* induce apoptosis during infection of macrophages by secreting IpaB and SipB proteins into the macrophage cytosol where they bind the proapoptotic protease caspase-1 to trigger apoptosis (Hilbi *et al.*,

1998; Hersh *et al.*, 1999). Micromolar concentrations of outer-membrane porin protein have been shown to induce apoptosis and necrosis in epithelial cells derived from rat seminal vesicle secretory epithelium (Buommino *et al.*, 1999). The type III secretion mechanism, whereby cytotoxic effector proteins are directly secreted into the host cell cytoplasm on contact (Frank, 1997), plays a major role in triggering apoptosis in macrophages and epithelial cells (Hauser & Engel, 1999) and also in human polymorphonuclear neutrophils (Dacheux *et al.*, 1999), although macrophages and epithelial cells respond differently to the *Pseudomonas aeruginosa* type III secretion system (Coburn & Frank, 1999). Such induction of apoptosis, however, requires cell-cell contact between the host and the pathogen.

We have recently reported that mucoid strains of *P. aeruginosa* isolated from cystic fibrosis (CF) patients secreted a number of ATP-utilizing enzymes that appeared to modulate the biotransformation of ATP effluxed from macrophages. Such modulation of the nature and concentration of ATP allowed activation of purinergic receptors, such as macrophage surface-associated P2Z receptors, which led to macrophage cell death through a change in cell permeability and vacuolization (Zaborina *et al.*, 1999). Interestingly, the nonmucoid laboratory strain PAO1 did not appear to secrete much of the ATP-utilizing enzymes and its cell-free supernatant showed low cytotoxicity towards macrophages. The mucoid *P. aeruginosa* strain 8821M was, however, shown not only to secrete ATP-utilizing enzymes that appeared to enhance the P2Z-receptor-mediated macrophage cell death, but also other cytotoxic factors that caused macrophage cell death through a P2Z-receptor-independent pathway (Zaborina *et al.*, 1999).

The studies by Zaborina *et al.* (1999) raised two interesting questions. Is secretion of ATP-utilizing enzymes and associated cytotoxic agents restricted to CF isolate mucoid strains, or is strain PAO1 an anomaly and other nonmucoid strains, particularly other nonmucoid clinical strains, demonstrate secretion of ATP-utilizing enzymes and non-P2Z-receptor-related cytotoxic factors? Secondly, what is the nature of the pathway that promotes macrophage cell death through the P2Z-receptor-independent pathway? In this paper, we demonstrate secretion of ATP-utilizing enzymes by a nonmucoid clinical *P. aeruginosa* isolate, strain 808, and provide evidence for the secretion of cytotoxic agents that induce apoptosis in macrophages in the absence of exogenous ATP. Usually, macrophage cell death mediated through activation of P2Z receptors is due to formation of pores in the cell membrane leading to necrosis; high concentrations (3 mM) of ATP have, however, been shown to induce apoptosis in myeloid cells mediated via activation of caspases such as caspase 1, 3 and 8 (Ferrari *et al.*, 1999). We report that two secreted redox proteins, azurin and cytochrome c_{551} , induce mast cell apoptosis in the absence of exogenous ATP.

METHODS

Strains, media and culture conditions. *P. aeruginosa* strain 808 is an isolate from a burn patient. Growth conditions, composition of the media and isolation of cell-free supernatants after centrifugation and filtration through a 0.22 µm filter were described previously (Zaborina *et al.*, 1999).

Assay for ATP-utilizing enzymes. The detection of adenine nucleotides by TLC followed by radioautography was described previously (Zaborina *et al.*, 1999).

Column chromatographic fractionation. The supernatant containing various cytotoxic factors was fractionated on hydroxyapatite, ATP-agarose and Q-Sepharose columns. Supernatant of strain 808 (2.0 l) grown on L broth to an OD₆₀₀ of 1.2 was filtered through a 0.22 µm filter and concentrated using an Amicon YM-10 membrane filter, followed by equilibration with 5 mM phosphate buffer pH 7.0. The concentrated supernatant was then loaded on a hydroxyapatite column (26 × 120 mm) equilibrated with the same buffer. The flow-through fraction was collected, equilibrated with TM (50 mM Tris/HCl pH 7.5 and 10 mM MgCl₂) buffer and loaded onto an ATP-agarose column (26 × 40 mm) equilibrated with the same buffer. The flow-through fraction was directly loaded onto a Q-Sepharose column (26 × 120 mm). The flow-through fraction from the Q-Sepharose column was concentrated and used for cytotoxicity assay. Part of this fraction was run on an SDS-PAGE gel, followed by transfer to PVDF membrane for N-terminal amino acid analysis using an automated Edman AB1477A protein sequencer (Applied Biosystems).

Macrophage cytotoxicity assays. The culturing of J774 macrophage cell line and the estimation of macrophage cell death by the release of lactate dehydrogenase (LDH) have been described previously (Zaborina *et al.*, 1999).

Confocal microscopy. The macrophage cells were cultured in a 0.15 mm thick dTC3 dish (Biotech) and treated with Q-Sepharose fraction for 6 or 16 h. Macrophage cells without any treatment were used as control. To detect changes in mitochondrial membrane potential and consequent macrophage apoptotic death during early stages of apoptosis (Green & Reed, 1998), an ApoAlert Mitochondria Membrane Sensor kit from Clontech was used. After treatment, the dish was placed on a temperature-controlled stage at 37 °C (Biotech Live Cell System) and images were obtained using a Carl Zeiss LSM510 laser scanning confocal microscope equipped with a ×25 objective. Beams (488 and 568 nm) from an argon-krypton laser were used for excitation, and green and red fluorescence emissions were detected through LP505 and LP560 filters, respectively.

Preparation of cytosolic macrophage extract. The cytosolic macrophage extract was prepared as described by Ellerby *et al.* (1997) and Stennicke *et al.* (1998). Briefly, 20 ml macrophage culture media in a plate was removed. Ice-cold Dulbecco's PBS pH 7.2 (Gibco BRL; 10 ml), was added to the plate. The cells were lifted gently off the plate, placed on ice in a 50 ml centrifuge tube and centrifuged (4 °C) at 200 g for 5 min. The resulting cell pellet was washed in 50 ml ice-cold PBS. The cells were resuspended in a 15 ml centrifuge tube with 10 ml of hypotonic extraction buffer (HEB) containing 50 mM PIPES pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂ and 1 mM DTT. The cells were centrifuged at 1000 g (4 °C) for 15 min to form a tight pellet. The supernatant was aspirated and HEB was added to an equal volume of the pellet. The mixture was allowed to stand on ice for 30 min. The cells

were then broken by passage through a 24-gauge needle (the desired extent of lysis, more than 90 %, was monitored under the microscope by trypan blue staining) and pelleted by centrifugation at 16000 *g* at 4 °C. The clarified supernatant (cytosolic extract) was removed carefully and was either used immediately or stored in aliquots at -84 °C. If stored, it was clarified by centrifugation just before use.

Haem staining. Haem staining in the Q-Sepharose protein fraction P3 was performed in gel (Goodhew *et al.*, 1986) using 3,3',5,5'-tetramethylbenzidine (Sigma).

RESULTS

Secretion of ATP-utilizing enzymes by nonmucoid *P. aeruginosa* strain 808

We previously reported that mucoid CF isolate strains of *P. aeruginosa* secrete various ATP-utilizing enzymes, such as 5'-nucleotidase, ATPase, nucleoside diphosphate kinase (Ndk) and adenylate kinase (Ak), whilst the nonmucoid laboratory strain PAO1 secreted very little of these enzymes (Zaborina *et al.*, 1999). The amounts of the secreted enzymes appeared to correlate with the level of cytotoxicity exhibited by these cell-free supernatant samples, suggesting that the secreted ATP-utilizing enzymes allowed biotransformation of ATP to various adenine nucleotides that in turn activated P2-purinergeric receptors on the surface of the macrophage. Activation of P2-purinergeric receptors (Dubyak & El-Moatassim, 1993), particularly the P2Z (P2X₇) receptors, cause macrophage cell death through permeabilization of hydrophilic molecules of up to 900 Da from the cell (Di Virgilio, 1995; Lammas *et al.*, 1997). Thus secretion of ATP-utilizing enzymes by mucoid *P. aeruginosa* cells appears to be one way in which the mucoid cells could subvert the host cell defence. To investigate whether nonmucoid non-CF isolate strains in general, similar to strain PAO1, lack this secretory property, we tested the ability of a number of mucoid and nonmucoid clinical isolates of *P. aeruginosa* to secrete both ATP-utilizing enzymes and cytotoxic factors against macrophages. Out of five nonmucoid (two environmental and three clinical) isolates, the two environmental isolates did not exhibit any ATP-utilizing enzymes or cytotoxicity (in the presence or absence of ATP) in their growth media. Two of the clinical isolates, however, demonstrated secretion of ATP-utilizing enzymes and ATP-enhanced cytotoxicity towards macrophages. Three mucoid CF isolates tested demonstrated the presence of ATP-utilizing enzymes as well as ATP-inducible cytotoxicity in their growth media. One nonmucoid clinical isolate from a burn patient, strain 808, proved to be a potent secretor of ATP-utilizing enzymes. Previously, the ATP-utilizing enzymes were assayed by incubating the cell-free growth medium of mucoid strain 8821M with [γ -³²P]ATP in presence of AMP or nucleoside diphosphates (NDPs) such as GDP, CDP and UDP. The presence of Ak was detected by the conversion of nonradioactive AMP to [³²P]ADP since Ak catalyses the reversible terminal phosphotransfer reaction $\text{AMP} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \rightleftharpoons [\text{P}^{32}]\text{ADP} + \text{ADP}$. The presence of Ndk could be detected

by the terminal phosphotransfer from [γ -³²P]ATP to GDP, CDP or UDP to form the corresponding nucleoside triphosphate (NTP). A nucleotidase (phosphatase) action was detected by the formation of NDPs and nucleoside monophosphates (NMPs) from NTPs or the release of ³²P_i (inorganic orthophosphate) from [α -³²P]ATP, [α -³²P]CTP or [α -³²P]GTP (Zaborina *et al.*, 1999). To detect the secretion of these enzymes, strain 808 was grown in tryptone-yeast extract (TYE) broth on a shaker at 37 °C for various times, aliquots were withdrawn every hour for 7 h, centrifuged to remove the cells, filtered through a 0.22 μm filter to remove residual cells, and the cell-free supernatant was tested for the presence of ATP-utilizing enzymes. As can be seen from the results shown in Fig. 1(a), Ak activity was detectable soon after inoculation and continued to rise throughout the 7 h growth period. Coincidental with growth (Fig. 1e) and increasing Ak activity, the release of P_i also increased, suggesting the secretion of an ATPase/nucleotidase activity (Fig. 1a). The detection of these activities at an early (exponential) phase of growth (2–3 h) is different from the case of the mucoid strain 8821M, where these activities were detectable only at high cell density when the cells entered stationary phase (Zaborina *et al.*, 1999). The detection of Ndk activity, as judged by the formation of [³²P]CTP from non-radioactive CDP (Fig. 1b), or a nucleotidase activity, as judged by the formation of [³²P]ADP from [α -³²P]ATP or [³²P]CDP from [α -³²P]CTP (Fig. 1c, d) during early to mid-exponential phase of growth (2–4 h) clearly indicated that nonmucoid strain 808 differed substantially from the mucoid strain 8821M with respect to early secretion of the ATP-utilizing enzymes.

We previously noted that the secretion of the ATP-utilizing enzymes by the mucoid strain 8821M was dependent on media composition. Secretion occurred only when the cells were grown in a medium with eukaryotic proteins such as casein or yeast extract (TYE broth). Very little secretion took place when the cells were grown in a basal synthetic medium (BSM) without any proteins (Zaborina *et al.*, 1999). To examine whether secretion from strain 808 was similarly dependent on growth in a complex medium, strain 808 was grown both in TYE broth and in BSM under identical conditions, aliquots were taken at various optical densities, centrifuged, filtered through 0.22 μm filters and the supernatants were tested for the presence of Ak by incubating the supernatant samples with [γ -³²P]ATP + 0.1 mM AMP. The results (Fig. 2) clearly showed that whilst Ak activity was detectable in the supernatants of the TYE-broth-grown cells, very little activity was detectable in that of BSM-grown cells, even at high cell density. The same results were obtained for other enzymes, such as Ndk and 5'-nucleotidase.

Enhancement of ATP-induced cytotoxicity in macrophages by the cell-free supernatant samples

Macrophages are known to efflux ATP on contact with cell wall LPS or intact bacteria (Ferrari *et al.*, 1997; Sikora *et al.*, 1999). Secretion of ATP-utilizing

Fig. 1. Assay of various ATP-utilizing enzymes secreted by *P. aeruginosa* strain 808. (a–d) After growth in L broth for various time periods as indicated, an aliquot of the growth medium was centrifuged to remove cells, filtered through a 0.22 μm filter and the supernatant was incubated with appropriate substrates. The separation and identification of various radioactive products of the reaction have been described previously (Zaborina *et al.*, 1999). (a) Detection of Ak activity as revealed by the formation of $[\gamma\text{-}^{32}\text{P}]\text{ADP}$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + AMP. Release of increasing amounts of inorganic phosphate ($^{32}\text{P}_i$) with increasing growth period is an indication of the presence of ATPase and/or nucleotidase/phosphatase; control $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + nonradioactive AMP is shown in lane C. (b) Detection of Ndk, as revealed by the formation of $[\gamma\text{-}^{32}\text{P}]\text{CTP}$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + CDP. (c) Detection of ATPase/nucleotidase activity as revealed by the formation of $[\gamma\text{-}^{32}\text{P}]\text{ADP}$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; control $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is shown in lane C. (d) Detection of CTPase/nucleotidase activity as revealed by the formation of $[\gamma\text{-}^{32}\text{P}]\text{CDP}$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ + CDP. (e) Growth curve of *P. aeruginosa* strain 808.

enzymes by mucoid *P. aeruginosa* has previously been shown to allow biotransformation and alteration of adenine nucleotide levels, leading to activation of macrophage surface-associated P2Z receptors, which in turn cause macrophage cell death (Zaborina *et al.*, 1999). Since increasing growth of strain 808 allowed increasing secretion of ATP-utilizing enzymes into the outside medium, it was of interest to determine if the

supernatant samples from different periods of growth would exhibit different levels of cytotoxicity towards macrophages. The supernatants taken at early exponential to stationary phase had very little cytotoxicity by themselves (Fig. 3, bars 1–5). However, supernatants taken from mid-exponential to stationary phase had enhanced cytotoxicity in the presence of 1.0 mM ATP (Fig. 3, 2 + ATP to 5 + ATP), suggesting that the ATP-

Fig. 2. Secretion of ATP-utilizing enzymes by *P. aeruginosa* strain 808 during growth in TYE (tryptone-yeast extract) broth (resembling L broth) but not in basal synthetic medium (BSM). Only data for Ak are presented. Aliquots of strain 808 grown in TYE and BSM were taken at various OD_{600} values as indicated, centrifuged, filtered through a 0.22 μ m filter, and Ak activities were determined by incubating the filtrate in the presence of [γ - 32 P]ATP+AMP, as described in the legend to Fig. 1. A sample of [γ - 32 P]ATP+AMP without supernatant was run as a control (lane C).

Fig. 3. Enhancement of ATP-induced cytotoxicity in macrophages by cell-free supernatants of strain 808 obtained at different periods of growth. Strain 808 was grown in TYE broth and samples were withdrawn at the following OD_{600} values: 1, 0.08; 2, 0.57; 3, 0.82; 4, 1.48; 5, 1.9. The samples were centrifuged, filtered, concentrated 10-fold using a 10 kDa cut-off centriprep membrane filter and used for cytotoxicity assays against macrophages as described previously (Zaborina *et al.*, 1999). Macrophage cell death was triggered using 1.0 mM ATP and estimated by LDH release. Samples 1 to 5 were incubated with macrophages in the absence of 1.0 mM ATP (bars 1 to 5), and also in the presence of 1.0 mM ATP (bars 1+ATP to 5+ATP). Macrophage cell death due to 1.0 mM ATP alone is shown in the middle (ATP). Results shown are means $\pm$ SD of triplicate experiments. Each sample contained 2 μ g supernatant protein.

Fig. 4. Pretreatment of macrophages with oATP abolishes subsequent ATP-induced cell death. Macrophages were either pretreated or not pretreated with 1.0 mM oATP for 2 h as described previously (Zaborina *et al.*, 1999). Such pretreated or non-pretreated macrophages were subsequently incubated with 1.0 mM ATP in the absence or in the presence of 10-fold concentrated supernatant samples of strain 808, as described in the legend to Fig. 3. Macrophages were also treated with an equivalent amount of supernatant sample without ATP (808sup) to determine cytotoxicity associated with the supernatant samples. Results shown are means $\pm$ SD of triplicate experiments. Each sample contained 3.0 μ g supernatant protein.

utilizing enzymes present in the supernatants acted on the ATP to produce various products that had a much higher cytotoxicity than either the supernatant fractions or ATP itself (Fig. 3, ATP).

Macrophage cytotoxicity is most likely mediated via P2Z receptor activation

Since ATP is a major agonist for P2Z receptor activation, and since P2Z receptor activation is known to lead to macrophage cell death (Di Virgilio, 1995; Lammas *et al.*, 1997), we were interested in knowing if the enhancement of ATP-induced macrophage cell death by the supernatant fractions from strain 808 is due to stimulation of activation of the P2Z receptors. It is known that oxidized ATP (oATP) is an irreversible inhibitor of P2Z receptor activation (Murgia *et al.*, 1993), such that pretreatment of macrophages with oATP blocks further activation of the P2Z receptors by exogenous ATP (Murgia *et al.*, 1993; Zaborina *et al.*, 1999). We therefore pretreated the macrophages with 1.0 mM oATP for 2 h before further treatment with ATP or strain 808 supernatant fractions in the presence of ATP. As expected, pretreatment of the macrophages with oATP led to a blockage of the P2Z receptors so that further addition of exogenous ATP did not lead to significant macrophage cell death (Fig. 4, ATP+oATP). Similarly, pretreatment of the macrophages with oATP before exposure to ATP+strain 808 supernatant fractions greatly reduced macrophage cell death (Fig. 4, 808sup+ATP+oATP), suggesting that the supernatant-induced enhanced macrophage killing

in the presence of ATP was largely due to activation of the macrophage surface-associated P2Z receptors.

P2Z-receptor-independent macrophage killing by fractionated supernatant: induction of apoptosis in macrophages

We previously reported that the cell-free supernatant fractions from mucoid CF isolate strain 8821M exhibited two types of cytotoxicity towards macrophages. During fractionation of the supernatant on hydroxyapatite or ATP-agarose columns, the flow-through fractions exhibited macrophage cytotoxicities that were stimulated in the presence of ATP and blocked when macrophages were pretreated with oATP. In contrast, during fractionation on a Mono Q column, the flow-through fraction was relatively devoid of the cytotoxic factors that operated through the P2Z receptor activation pathway, and thereby became enriched with cytotoxic factors that were less stimulated in the presence of ATP and were relatively independent of pretreatment of the macrophages with oATP (Zaborina *et al.*, 1999). This suggested the presence of an additional cytotoxic factor(s) that exerted its action independent of P2Z receptor activation. Nothing was, however, known about the nature of this additional cytotoxic factor(s). In an effort to see if strain 808 might produce similar P2Z-receptor-independent cytotoxic factors, we fractionated the supernatant samples of strain 808 through hydroxyapatite, ATP-agarose and Q-Sepharose columns. The flow-through fractions of hydroxyapatite and ATP-agarose columns had low cytotoxicity by themselves (Fig. 5b, HA and ATPag), but their cytotoxicities increased substantially in the presence of ATP, suggesting mediation via P2Z receptor activation (data not shown). In contrast, the flow-through fraction from the Q-Sepharose column was enriched with a cytotoxic factor(s) (the P2Z-receptor-dependent cytotoxic factors being largely retained in the column) that was independent of exogenous addition of ATP (Fig. 5b, Qseph and Qseph+ATP) and appeared to operate through a P2Z-independent pathway.

To estimate the number of major proteins present in various fractions, a part of each sample was run on an SDS-PAGE gel and the number of protein bands visualized by Coomassie blue staining. As shown in Fig. 5a, the supernatant fraction (super) showed the presence of small amounts of a number of bands. The hydroxyapatite flow-through fraction (HA) also showed a number of bands while the ATP-agarose flow-through fraction (ATPag) showed four major bands with a few minor species. In contrast, the Q-Sepharose column flow-through fraction (Qseph), which showed ATP-independent cytotoxicity, harboured three major protein bands labelled P1, P2 and P3. The N-terminal sequence of P1 and P2 suggested that they were elastase and azurin. The P3 protein has not been fully characterized but it appears to be a redox protein of cytochrome *c* type with a haem group, most probably cytochrome *c*₅₅₁ (data not shown). Azurin is a copper-

Fig. 5. (a) SDS-PAGE separation of proteins present in the flow-through fractions of various columns and their detection by Coomassie blue staining. Total proteins (20 µg) from the supernatant (super), and from the hydroxyapatite (HA) and ATP-agarose (ATPag) column flow-through fractions was loaded in the wells, and 6.0 µg Q-Sepharose (Qseph) flow-through fraction was used for detecting protein bands. Molecular mass standards are shown in lane M. The N-terminal amino acid sequences of bands P1 and P2, representing elastase and azurin, are shown on the right. (b) Fractionation of the strain 808 supernatants on various columns and enrichment of a fraction after Q-Sepharose column chromatography that demonstrates ATP-independent cytotoxicity. Concentrated supernatant from strain 808 was fractionated on hydroxyapatite, ATP-agarose and Q-Sepharose columns as described in Methods. The flow-through fractions from each column were then tested for cytotoxicity towards macrophages, estimated by LDH release. The supernatant (super), and the flow-through fractions from the hydroxyapatite (HA) and ATP-agarose (ATPag) columns had low cytotoxicity by themselves as shown, but high cytotoxicity in the presence of ATP (not shown). In contrast, the flow-through fraction from the Q-Sepharose column (Qseph) had high cytotoxicity by itself, whilst 1.0 mM ATP demonstrated about 20% macrophage cell death (ATP). Addition of exogenous ATP (1.0 mM) to the Q-Sepharose flow-through fraction (Qseph+ATP) did not lead to additional cell death. Results shown are means ± SD of triplicate experiments.

containing redox protein, originally believed to be involved in dissimilatory nitrate reduction, but has subsequently been shown not to be essential for denitrification (Vijgenboom *et al.*, 1997). Cytochrome *c*₅₅₁ is known to co-purify with azurin and participates in the electron-transfer reaction to azurin during denitrification (Van de Kamp *et al.*, 1990).

Fig. 6. Detection of caspase-3 activity in cytosolic extracts of macrophages grown in the absence or presence of 10 µg Q-Sepharose flow-through fraction ml⁻¹. Macrophages (10⁷ cells) derived from the cell line J774 were grown in RPMI-1640 medium with 10% foetal bovine serum and incubated overnight with or without Q-Sepharose flow-through fraction. The macrophage cells were then broken and cytosolic extracts were prepared as described in Methods. Caspase-3 activity was assayed at 37 °C using 200 µg cytosolic protein in 100 µl reaction mixture containing the colorimetric substrate Ac-DEVD-pNa (100 µM), as described by Stennicke & Salvesen (1999). pNa (p-nitroaniline) release was measured at 405 nm. Line 1, cleavage of caspase-3 substrate by cytosolic extracts of macrophages not treated with Q-Sepharose flow-through fraction; line 2, above extract incubated in presence of 5 µg Q-Sepharose flow-through fraction; line 3, above extract incubated in presence of 5 µg Q-Sepharose flow-through fraction + 10 mM dATP; line 4, above extract incubated in presence of 10 µM cytochrome *c* + 10 mM dATP; line 5, cleavage of caspase-3 substrate by cytosolic extract from macrophages incubated overnight with Q-Sepharose flow-through fraction.

Induction of apoptosis in macrophages is mediated via caspase-3

We considered the possibility that the ATP-independent macrophage killing by the Q-Sepharose flow-through fraction is mediated by triggering of apoptosis through caspase activation. Caspases are cysteine proteases cleaved at aspartic residues and exist as inactive procaspase forms predominantly in the cytosol. Caspase-3 is a key downstream caspase of the caspase cascade, which is activated in apoptotic mammalian cells (Green & Reed, 1998). Since apoptotic cells demonstrate the presence of active caspases, for some of which substrates are available (Stennicke & Salvesen, 1999), we determined the presence of active caspase-3 in the cytosolic extract of murine J774 macrophage cells using the synthetic tetrapeptide substrate Ac-DEVD-pNa (N-acetyl-Asp-Glu-Val-Asp-*p*-NO₂-aniline). As can be seen from the results shown in Fig. 6, the macrophage cytosolic extract had very little caspase-3 activity (line 1), suggesting that most of the caspase-3 was present as inactive procaspase-3. Addition of 5 µg Q-Sepharose column flow-through fraction in the ab-

sence (line 2) or in the presence of 10 mM dATP (line 3) did not show activation of the inactive procaspase-3 to caspase-3. Addition of 10 µM cytochrome *c* and 10 mM dATP, however, allowed a steady activation of the procaspase-3 to caspase-3, which became detectable in 60 min (line 4). Cytochrome *c* is an important component of the apoptotic process since, on receiving the death signal, it is released from mitochondria to the cytosol where it forms a complex with a cytosolic protein, Apaf-1; in the presence of dATP, this complex allows activation of procaspase-9 to caspase-9, which in turn activates caspase-3 (Zou *et al.*, 1997, 1999). Our data suggested that the Q-Sepharose column flow-through fraction, unlike cytochrome *c*, could not directly activate caspase-3 in the macrophage cytosolic fraction. However, when the macrophages were incubated overnight in the presence of a similar amount of Q-Sepharose flow-through fraction, and cytosolic extracts were made and assayed for caspase-3 activity, significant caspase-3 activity was detected (Fig. 6, line 5), suggesting that apoptosis was triggered in intact macrophages when they were subjected to the cytotoxic factors present in the Q-Sepharose flow-through fraction.

To confirm that induction of caspase-3 activity in macrophages exposed overnight to the Q-Sepharose column flow-through fraction is indeed due to apoptosis, we attempted to detect apoptotic macrophages as a function of contact time with the strain 808 cytotoxic factor(s). For this purpose, we used Clontech's ApoAlert Mitochondrial Membrane Sensor kit, which uses a cationic dye (Mitosensor) that produces red fluorescence in healthy, nonapoptotic cells because of mitochondrial Mitosensor aggregate formation. In apoptotic cells, Mitosensor cannot aggregate in the mitochondria because of altered mitochondrial membrane potential, and remains in the cytoplasm as monomers exhibiting green fluorescence. In transitional cells undergoing apoptosis, a combination of red and green fluorescence causes cells to appear yellow. When macrophages were incubated overnight in the absence of the Q-Sepharose flow-through fraction and tested with the ApoAlert Mitosensor kit, they fluoresced red, indicative of a nonapoptotic, healthy state (Fig. 7a). When the macrophages were incubated for 6 h in the presence of the Q-Sepharose flow-through fraction, many macrophages fluoresced green, indicating that apoptosis had set in (Fig. 7b). When the macrophages were incubated overnight with the Q-Sepharose flow-through fraction, there were very few healthy (red-fluorescing) cells; most of the cells were green-fluorescing (Fig. 7c) demonstrating that the Q-Sepharose flow-through fraction triggered extensive apoptosis in macrophages during overnight exposure.

Induction of apoptosis by azurin and cytochrome *c*₅₅₁ in murine mast cells

Our demonstration that the Q-Sepharose flow-through fraction triggered apoptosis in cell-line-J774-derived macrophages raised the question of whether induction

Fig. 7. Detection of apoptotic macrophage cells on incubation with Q-Sepharose flow-through fractions for 6 or 16 h, using an ApoAlert Mitochondria Membrane Sensor kit. (a) Macrophages derived from cell line J774 were rinsed with serum-free media, stained with Mitosensor at 37 °C for 15 min, rinsed with incubation buffer and analysed by confocal microscopy as detailed in Methods. (b) Macrophages were treated with 10 µg Q-Sepharose flow-through fraction for 6 h prior to staining with Mitosensor. (c) Macrophages were incubated with 10 µg Q-Sepharose flow-through fraction for 16 h before staining with Mitosensor. Since after staining with Mitosensor the macrophages are washed to remove the dye, many macrophages undergoing apoptosis do not adhere very well and are washed off, giving a low count of apoptotic cells.

Fig. 8. Induction of mast cell cytotoxicity by the Q-Sepharose flow-through fraction (Qseph fraction; 18 µg protein), buffer control (buffer) and various concentrations of azurin (Az) and/or cytochrome c_{551} (Cytc551). Numbers in parentheses represent µg Az or cytochrome c_{551} used in each assay. Details of the preparation of mast cells and LDH release assay have been described previously (Melnikov *et al.*, 2000; Punj *et al.*, 2000). Results shown are means $\pm$ SD of triplicate experiments.

of apoptosis is limited to such macrophages or may extend to primary macrophages such as murine peritoneal macrophages. Consequently, we tested the effect of the Q-Sepharose flow-through fraction on peritoneal macrophages, treated with or without bacterial cell wall LPS. The Q-Sepharose fraction caused extensive cell death to peritoneal macrophages either treated or not treated with LPS in the absence or in the presence of ATP (data not shown). Since the Q-Sepharose fraction was enriched with azurin and cytochrome c_{551} (Fig. 5a), and to investigate whether induction of apoptosis may be true of other phagocytic cells such as mast cells, we incubated murine mast cells overnight with the Q-

Sepharose flow-through fraction or various concentrations of purified azurin and cytochrome c_{551} , singly or in combination. The extent of mast cell death was then determined by LDH release (Fig. 8), as well as Mitosensor screening (data not shown). Whilst azurin was partially active and cytochrome c_{551} was poorly active in causing mast cell death, a combination of azurin and cytochrome c_{551} triggered extensive cell death (Fig. 8) through induction of apoptosis as measured by altered mitochondrial membrane permeability detected by the Mitosensor fluorescence technique (data not shown). Thus secreted azurin and cytochrome c_{551} play a significant role in the induction of phagocytic cell apoptosis, although the mechanism of induction of apoptosis by these two bacterial redox proteins is very different from that triggered by mitochondrial cytochrome c (Reed, 1997; Zou *et al.*, 1999).

DISCUSSION

Pathogens tend to use multiple pathways to subvert the host defence. Thus it is not surprising that *P. aeruginosa* uses both an ATP-inducible P2Z-receptor-mediated and a caspase-3 mediated pathway to enhance macrophage cell death. Whilst induction of apoptosis by *P. aeruginosa* cytotoxic factors has recently been reported (Hauser & Engel, 1999; Dacheux *et al.*, 1999), such factors are directly secreted into the host cell cytosol by a type III secretion system, thus requiring host-pathogen contact. In contrast, both the ATP-agarose flow-through fraction, which allows macrophage cell death by enhancing the ATP-P2Z receptor activation pathway, or the Q-Sepharose flow-through fraction, which primarily allows macrophage cell death by triggering the caspase-3-mediated apoptotic pathway, are free of whole cells. It should, however, be pointed out that secretion of these cytotoxic factors is dependent on the growth medium, which needs to contain eukaryotic proteins such as casein (tryptone, for instance; Zaborina *et al.*, 1999).

Vallis *et al.* (1999) have reported that ExoS induction could be observed when *P. aeruginosa* was in contact with Chinese hamster ovary (CHO) cells or after growth in tissue culture medium with serum. The serum induction of ExoS appeared to result in generalized type III secretion, whilst induction by contact with CHO cells appeared to result in polarized type III secretion. In the present case, strain 808 could secrete the cytotoxic factors whilst grown in TYE broth or L broth without serum. It would be interesting to see if mutants of strain 808 defective in the type III secretion pathway are still able to secrete the cytotoxic factors.

Since the secreted ATP-utilizing enzymes convert ATP to adenosine and various adenine nucleotides, the effective ATP concentration in the external milieu of the macrophages and mast cells should decrease, resulting in a loss of P2Z-receptor-mediated activation. Thus our observation of enhanced cytotoxicity in the presence of ATP by the supernatant fraction of strain 808 may seem to be something of a paradox. We previously reported that the mucoid CF isolate strain 8821 secretes a putative ATP reductase that changes the ionic state of ATP (Zaborina *et al.*, 1999). Although ATP is an agonist for P2Z receptor activation, various ionic forms of ATP, such as ATP⁴⁻ or benzoyl benzoyl ATP, are even better agonists (Di Virgilio, 1995). Thus the secreted ATP-utilizing enzymes may change the redox status of ATP, producing a better agonist than ATP itself and thereby enhancing macrophage cell death through increased P2Z receptor activation. We have also recently demonstrated that a mixture of ATP + ADP + AMP + adenosine at a combined concentration of 0.5 mM can enhance the cell death of peritoneal macrophages in the presence of cell-free growth medium of a clinical isolate of *Burkholderia cepacia* more than 0.5 mM ATP or other adenine nucleotides by themselves (Melnikov *et al.*, 2000). We postulated that various adenine nucleotides, produced by the action of secreted ATP-utilizing enzymes on ATP, activate various purinergic receptors, leading to enhanced phagocytic cell death (Melnikov *et al.*, 2000; Punj *et al.*, 2000). It is likely that many pathogens have evolved this efficient secretory system for the effective elimination of phagocytic cells.

The presence of redox proteins such as azurin and an unidentified 8–9 kDa protein with a haem group (protein P3) in the Q-Sepharose fraction is interesting. In the presence of apoptotic signals such as growth factor deprivation, DNA damage or activation of cell surface death receptors such as Fas and TNF (Salvesen & Dixit, 1997), caspases, which normally exist as inactive zymogens in the cytosol, become activated through proteolysis. Such activation during apoptosis is believed to occur through a caspase cascade, with caspase-3 playing a major role. Cytochrome *c*, which normally resides in the mitochondria, is released into the cytosol during apoptosis because of altered membrane permeability. In the presence of dATP, cytochrome *c* promotes multimerization of Apaf-1 to form a complex that can activate caspase-3 (Zou *et al.*, 1997), presumably through activation of caspase-9 (Zou *et al.*, 1999). Our

demonstration of the ability of *P. aeruginosa* redox proteins such as azurin and cytochrome *c*₅₅₁ to trigger apoptosis in macrophages and mast cells is another example of the role of redox proteins in mammalian cell apoptosis, although the mechanism of initiation of this apoptotic process seems to be different. Further investigations are under way to explore how the bacterial redox proteins are bound and internalized in macrophages to trigger their death.

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