Burkholderia cenocepacia Induces Neutrophil Necrosis in Chronic Granulomatous Disease

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Burkholderia cenocepacia complex is a life-threatening group of pathogens for patients with chronic granulomatous disease (CGD), whose phagocytes are unable to produce reactive oxygen species (ROS). Unlike other CGD pathogens, B. cenocepacia complex is particularly virulent, characteristically causing septicemia, and is the bacterial species responsible for most fatalities in these patients. We found that a nonmucoid Burkholderia cenocepacia (a predominant species in the B. cenocepacia complex) isolate was readily ingested by normal human neutrophils under nonopsonic conditions and promoted apoptosis in these cells. The proapoptotic effect was not due to secreted bacterial products, but was dependent on bacterial viability. Phagocytosis was associated with a robust production of ROS, and the apoptotic neutrophils could be effectively cleared by monocyte-derived macrophages. The proapoptotic effect of B. cenocepacia was independent of ROS production because neutrophils from CGD patients were rendered apoptotic to a similar degree as control cells after challenge. More importantly, neutrophils from CGD patients, but not from normal individuals, were rendered necrotic after phagocytosis of B. cenocepacia. The extreme virulence of B. cenocepacia complex bacteria in CGD, but not in immunocompetent hosts, could be due to its necrotic potential in the absence of ROS. The Journal of Immunology, 2005, 174: 3562–3569.

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency resulting from genetic defects in components of the phagocyte NADPH-oxidase, rendering the patient’s phagocytes unable to produce the reactive oxygen species (ROS) needed for proper antimicrobial activity (1). These patients suffer from recurrent infections with a distinct set of fungal and bacterial species and often display various inflammatory complications, including granuloma formation (2). The leading bacterial cause of CGD fatalities are members of the Burkholderia cepacia complex, a complex that includes nine distinct species (3). Burkholderia cenocepacia (formerly known as genomovar III) is a particularly problematic pathogen in patients with cystic fibrosis (CF) (4), but no particular species from the complex appears to predominate in CGD. B. cepacia complex bacteria possess intrinsic resistance to many antibiotics and can be transmitted from patient to patient in CF, making infection with these bacteria a particularly difficult clinical challenge. B. cepacia complex bacteria are highly resistant to cationic antibacterial peptides (5), crucial components of the oxygen-independent killing machinery of phagocytes, rendering these pathogens especially well suited for infection in CGD.

Their particular virulence in patients with CF has not been elucidated.

B. cepacia complex infections in CGD patients are often remarkably aggressive, and the majority of the fatalities are caused by bacteraemic spread, a condition extremely uncommon with other bacterial species. Furthermore, B. cepacia complex bacteria can cause septicemia in CF, a condition very rarely seen with the more common CF pathogen, Pseudomonas aeruginosa. This suggests that B. cepacia complex possesses virulence factors, making it unique among bacterial CGD and CF pathogens (4). In both CGD and CF, B. cepacia complex bacteria most commonly infect the respiratory tract, and the pathology is characterized by excessive inflammation with a massive infiltration of neutrophils.

Neutrophils are rapidly recruited to a site of infection, where they ingest and destroy the invading microorganisms using a combination of ROS and a variety of cationic peptides and proteolytic enzymes. Normally, after phagocytosis, the neutrophils undergo apoptosis (also known as programmed cell death) and are disposed of by other cell types, most commonly macrophages (6). ROS are not only crucial bactericidal effectors, but they are also important signaling molecules and are partly responsible for initiating the apoptotic program (7, 8). Antioxidants or inhibitors of the neutrophil NADPH-oxidase inhibit apoptosis following phagocytosis of a variety of bacterial species (7–10). In addition, neutrophils from CGD patients display delayed spontaneous apoptosis (11) as well as decreased apoptotic rates following ingestion of heat-killed Staphylococcus aureus (9). Yamamoto et al. (9) concluded that even though CGD neutrophils exhibited lower apoptotic levels than control cells, the ingestion of S. aureus still promoted apoptosis in these cells, indicating that ROS-independent pathways also play a role in phagocytosis-mediated apoptosis.

Apoptosis and subsequent clearance represent important physiological steps for terminating an inflammatory response. However, neutrophils can also undergo a less physiologic form of cell death: necrosis. Necrosis is a more pathological type of cell death in which the cell membrane loses its integrity, and leakage of cellular contents leads to cell swelling and eventual cell death.
contents occurs (12). If neutrophils die by necrosis, their toxic contents may leak out and cause additional tissue damage and perpetuation of the inflammatory reaction. Necrotic cell death (instead of apoptosis) could result in chronic inflammation rather than resolution of the inflammatory response (13).

We studied the interaction of human neutrophils with *B. cenocepacia* and investigated how this interaction affected neutrophil cell death. Furthermore, we evaluated whether ROS affected the outcome of neutrophil interaction with *B. cenocepacia*, thus providing clues as to why these bacteria display such extraordinary virulence in CGD. Our data demonstrate that a clinical *B. cenocepacia* isolate was readily ingested nonopsonically by neutrophils from normal individuals. Ingestion was accompanied by ROS production, and the interaction resulted in the induction of neutrophil apoptosis. Challenge of neutrophils from CGD patients with this isolate induced slightly higher levels of apoptosis than in normal neutrophils, suggesting that ROS-independent pathways are operative. More importantly, however, *B. cenocepacia* also induced significant levels of necrosis in CGD neutrophils, suggesting that ROS are crucial for controlling ingested *B. cenocepacia* and abrogating cytotoxic/necrotic effects. In summary, these results could explain why certain *B. cenocepacia* complex bacteria are capable of causing such severe, necrotizing infections in CGD patients while posing little or no threat to immunocompetent hosts.

**Materials and Methods**

**Patients**

Peripheral blood was obtained from healthy volunteers and three CGD patients, two male gp91phox-deficient patients (X-linked CGD) and one female gp91phox-deficient patient (autosomal recessive CGD). None of the patients was infected at the time of the experiments. All donors (healthy controls and CGD patients) provided informed consent, and the study was approved by the Clinical Research Ethics Board of the University of British Columbia.

**Isolation of cells**

Human neutrophils were purified using dextran sedimentation and Ficoll-Paque gradient centrifugation (14). The cells were washed and resuspended (10⁶/ml) in Krebs-Ringer phosphate buffer (KRG, pH 7.3) containing glucose (10 mM), Ca²⁺ (1 mM), and Mg²⁺ (1.5 mM), and stored on melting ice until use. This protocol routinely produced a neutrophil population of ~95% purity, as judged by visual inspection of Giemsa-stained slides. About 95% of the freshly isolated neutrophils were viable and negative for both annexin V and ethidium homodimer 1 (EthD). Human monocytic-derived macrophages (MDMs) were obtained as previously described (15), except that cells were allowed to differentiate in Teflon pots with 100 ng/ml M-CSF instead of autologous serum, and used for apoptotic neutrophil uptake assays on day 6.

**Bacterial strains and growth**

*B. cenocepacia*, strain C9863, was a nonmucoid, genomovar III-A B. cepacia complex isolate from a CF patient. The mucoid variant, strain C9343, was a sequential isolate from the same CF patient, and both strains have been previously described (16). Throughout the text, the term *B. cenocepacia* refers to the nonmucoid isolate C9863, and the mucoid variant is always referred to as mucoid *B. cenocepacia*. The isolates are deposited in the Canadian *B. cepacia* Complex Research and Referral Repository. The strains were stored at ~80°C or maintained on Columbia agar containing 5% sheep blood (PML Microbiologics) for a maximum of two passages. Unless otherwise specified, bacteria were grown in Luria broth at 37°C and 250 rpm overnight, washed, and cultured in KRG plus 0.3% BSA for 1 h until reaching the desired OD (corresponding to ~10⁶ CFU/ml). For some experiments, an aliquot (2 ml) was UV killed by incubation for 5 min in 24-well tissue culture plates placed on a UV table (UV Transilluminator; Ultraviolet Products), equipped with four 15-W lamps. Viable counts confirmed that treatment for 50 min reduced viability of strain C9863 by >99.99%. The NADPH-oxidase assay was performed on bacteria that were concentrated using a microcentrifuge (9000 × g, for 2 min) and resuspended to a density of 10⁶ CFU/ml in KRG (without BSA).

**LPS purification and generation of bacterial supernatants**

LPS was purified by suspending a freeze-dried bacterial pellet in an extraction mixture consisting of phenol (90%)/chloroform:petroleum spirit in the proportions 2:5:8. The mixture was stirred on ice for 10 min and centrifuged (10,000 × g, for 15 min), after which the supernatant was filtered. The filtrate was air dried and repurified (17), washed in ice-cold ethanol, and resuspended in endotoxin-free water plus 0.2% triethylamine after drying. When cell-free bacterial supernatants were used, the bacteria were grown in RPMI 1640 medium (without antibiotics) overnight and the supernatants were harvested by sedimenting out the bacteria in a microcentrifuge (9000 × g, for 2 min) and then filtered (0.2 µm) before use.

**Neutrophil NADPH-oxidase activity**

The NADPH-oxidase activity was determined using a luminol-ECL system (18) with a Victor² (PerkinElmer Life and Analytical Sciences) plate reader and disposable 96-well plates containing 220-µl reaction mixtures. Each well contained 10⁶ neutrophils, luminol (a cell-permeable chemiluminescence substrate; 2 × 10⁻³ M), and cell-impermeable scavengers superoxide dismutase (20 U) and catalase (2000 U). The plates were equilibrated in the Victor² for 10 min at 37°C, after which the stimulus (30 µl) was added. The light emission was recorded continuously, and data are expressed as counts per second.

**Bacterial challenge**

Freshly isolated neutrophils were diluted to 10⁷ cells/ml in KRG supplemented with 0.3% BSA (to minimize cellular aggregation and adhesion to the tubes) and incubated at 37°C for 10 min in the presence or absence of diphénylène iodotrim (DPI; final concentration 10⁻³ M) before bacteria were added, giving a final volume of 1 ml and a multiplicity of infection of 30 bacteria/neutrophile. Where indicated, cytochalasin D was included at a concentration of 10⁻⁶ M. All bacterial challenges took place in polypropylene tubes slowly tumbling at 37°C for 1 h, after which the cells were subjected to repeated washes in PBS (110 × g, for 10 min, to remove nonattached bacteria) and resuspended in 0.5 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 250 µg/ml meropenem (RPMI 1640 medium), and DPI (10⁻³ M) when required. Samples were withdrawn for cytospinning, and the tubes were incubated standing at 37°C in a humidified CO₂ incubator (5% CO₂, 95% air). After 18–20 h of incubation, the supernatants were collected and the cells were washed in PBS before FACS analysis.

**Lactate dehydrogenase (LDH) assay**

Supernatants (300 µl) were spun in a microcentrifuge, and 250 µl of cell-free supernatant was analyzed by a cytotoxicity detection kit (Roche Applied Science) to quantify released levels of LDH, according to the manufacturer’s instructions. In parallel with the bacterially challenged samples, control samples including medium only (blank) and 2.5 × 10⁷ neutrophils in 1% Triton X-100 (total) were also analyzed, and data are presented as LDH released as percentage of total LDH after subtraction of blank sample readings.

**Flow cytometric measurements of neutrophil cell death**

Washed neutrophil cell pellets were resuspended in 300 µl of annexin V-binding buffer containing 5 µl of Annexin V-FITC (BD Biosciences) and 5 µM EthD (Molecular Probes). EthD is a cell-impermeable nucleic acid-binding dye that only permeates leaky (necrotic) cell membranes and detects necrotic cell death (19). The cells were incubated in the dark for 15 min, and analysis was performed on >10,000 cells using a FACSCalibur system and CellQuest software (BD Biosciences). Neutrophils were gated to exclude cellular debris and unbound bacteria, the amount of annexin V (FL1) and EthD (FL3) binding to the cells was measured, and the data were analyzed using WinMDI 2.8 software, defining cells as viable (annexin V⁻EthD⁻), apoptotic (annexin V⁻EthD⁻), or necrotic (annexin V⁺EthD⁻).

**Neutrophil phagocytosis**

After cytospinning, slides were air dried overnight, fixed in methanol (5 min), Giemsa stained, and mounted. Slides were studied using oil immersion and ×1000 magnification; >200 cells were scored as either positive or negative for bacterial association, and the number of bacteria/cell was counted for the first 5 positive cells. Phagocytosis is expressed as mean number of bacteria (bound or ingested)/cell.
MDM uptake of apoptotic neutrophils

Freshly prepared neutrophils were labeled using Vybrant CFDA SE cell tracker kit (Molecular Probes), according to the manufacturer’s instructions, using a dye concentration of 1 μM. Fluorescent cells were washed in PBS and either subjected to challenge with *B. cenocepacia* (as above) or resuspended in RPMI 1640 medium supplemented with 1 μg/ml FAS ligand (mouse anti-human CD95; eBiosciences) and 1 μg/ml cross-linking Ab (anti-mouse IgM; eBiosciences) to induce apoptosis. After overnight incubation, the apoptotic cells (levels of apoptosis were analyzed morphologically and were ~70% for both conditions in the experiment shown) were washed and resuspended in HBSS plus 0.1% gelatin.

MDMs were washed and resuspended in HBSS plus 0.1% gelatin before mixing with apoptotic neutrophils in polypropylene tubes (10^5 MDMs and 10^6 neutrophils in 400 μl) in the presence or absence of cytochalasin D (5 μg/ml incubated with the MDMs for 15 min at 37°C before addition of neutrophils) and left in a CO2 incubator at 37°C for 90 min. After washing, the MDMs were stained using an anti-CD14 allophycocyanin Ab (Caltag Laboratories) 1 h on ice and analyzed by flow cytometry. Samples were first gated based on forward scatter vs side scatter, and the gate was set around MDMs using an MDM-only sample (cells within this gate were >90% positive for CD14). This gate also included occasional neutrophils and was used to plot CFSE vs CD14. During analysis, cells were defined as CD14-positive cells also expressing CFSE fluorescence in the presence or absence of cytochalasin D. After FACS analyses, the remaining cells were cytospun, stained with 4',6'-diamidino-2-phenylindole (DAPI), mounted, and inspected visually using a Zeiss Axioplan 2 microscope with FITC and DAPI fluorescence filters.

Results

**Neutrophil uptake of *B. cenocepacia***

To study the initial interaction between neutrophils and *B. cenocepacia*, neutrophils purified from peripheral blood of healthy volunteers were coincubated with the nonmucoid *B. cenocepacia* isolate, C8963, for 1 h. After challenge, the cells were washed at low speed to remove the majority of noningested bacteria, and the uptake of bacteria was determined microscopically. The bacteria were readily taken up by the neutrophils under nonopsonic conditions, and most cells had ingested multiple bacteria. Fig. 1A shows neutrophils containing multiple ingested bacteria, many of which resided within phagosome-like organelles (arrows). When the bacteria were UV killed before challenge, the level of phagocytosis was not significantly different from that obtained using viable bacteria (Fig. 1B). Phagocytosis was not affected by the presence of DPI, an inhibitor of the neutrophil NADPH-oxidase (Fig. 1B).

**Neutrophil apoptosis following phagocytosis***

After bacterial challenge, cells were washed and incubated in RPMI 1640 medium supplemented with bacteriostatic concentrations of meropenem. After 18 h of incubation, cells were washed and stained with annexin V and the cell-impermeable nucleic acid-binding dye EthD, to assess apoptosis and necrosis. Approximately 30–50% of untreated cells were annexin V-positive/EthD-negative, representing spontaneous levels of apoptosis. A significantly

![FIGURE 1.](http://www.jimmunol.org/) Nonopsonic interaction of *B. cenocepacia* and human neutrophils. A, Giemsa-stained slides of neutrophils after 1-h nonopsonic interaction with viable *B. cenocepacia*. Arrows indicate bacteria residing in phagosome-like organelles. B, Cellular association with bacteria (viable or UV killed) in the presence or absence of DPI as determined by microscopic evaluation and is expressed as mean number of bacteria (bound or ingested)/cell + SEM of three to six independent experiments.

![FIGURE 2.](http://www.jimmunol.org/) Viable, but not UV-killed, *B. cenocepacia* induce neutrophil apoptosis. A, Effect of challenge with viable *B. cenocepacia* on neutrophil apoptosis assessed by annexin V binding alone or in combination with EthD, or by surface expression of CD16. Representative experiments are shown. B, The effect on neutrophil apoptosis by challenge with viable or UV-killed bacteria, as determined by flow cytometry. Apoptotic cells were defined as annexin V high/EthD low. Data are mean + SEM of three to seven independent experiments, and asterisks indicate statistically significant differences (p < 0.05).
higher proportion of bacterially challenged neutrophils was apoptotic compared with unchallenged cells (Fig. 2). Annexin V binding is but one characteristic feature of apoptotic neutrophils; other characteristics include down-regulation of certain cell surface receptors and DNA fragmentation, followed by nuclear condensation (20). The proapoptotic effect of this B. cenocepacia strain was confirmed also by microscopic evaluation of cellular morphology (data not shown) and loss of surface-located CD16 (Fig. 2A). When the bacteria were UV-killed before challenge, no induction of apoptosis was seen; instead, UV-killed bacteria had slight antiapoptotic effects (not statistically significant), indicating that the proapoptotic effect was dependent on bacterial viability (Fig. 2B). The proportion of necrotic neutrophils (i.e., positive for both annexin V and EthD) was low (~5%) irrespective of bacterial challenge.

**Antiapoptotic effects of bacterial-derived molecules**

Previous studies have shown that B. cepacia complex can secrete a hemolysin that induces apoptosis in human neutrophils (21). To investigate whether the proapoptotic effect was due to secreted bacterial products, neutrophils were incubated in the presence of cell-free bacterial supernatants. Instead of inducing apoptosis, however, bacterial supernatants had a marked antiapoptotic effect and inhibited spontaneous apoptosis in a dose-dependent manner (Fig. 3). Purified LPS from the B. cenocepacia isolate also was antiapoptotic in a dose-dependent manner in the nanogram per milliliter range (Fig. 3). Thus, we concluded that no soluble factors released from the bacteria were responsible for the induction of apoptosis, and that released factors, such as LPS, instead constituted potent survival signals. That B. cenocepacia induced neutrophil apoptosis suggested that the uptake of viable bacteria was a proapoptotic event capable of overriding the effects of antiapoptotic molecules released from the bacteria.

**Neutrophil apoptosis is dependent on bacterial uptake**

A mucoid B. cenocepacia variant, previously shown to be genetically very closely related to the nonmucoid strain C8963, interacted significantly less with human neutrophils than the nonmucoid isolate (16). When this mucoid strain was used to challenge neutrophils, very low levels of association were measured, and in contrast to the nonmucoid isolate, association was not inhibitable by cytochalasin D (Fig. 4A), indicating that the mucoid strain was not phagocytosed. Challenge with the mucoid isolate did not induce neutrophil apoptosis; on the contrary, it protected cells from spontaneous apoptosis (Fig. 4B). LPS and supernatant from the mucoid strain were also potently antiapoptotic (data not shown), indicating that in the absence of phagocytosis, antiapoptotic molecules secreted from the bacteria constituted powerful survival signals.

**Clearance of apoptotic neutrophils**

Apoptotic cells are normally cleared through uptake by other, viable phagocytes, in particular macrophages (13). To determine whether neutrophils rendered apoptotic by phagocytosis of nonmucoid B. cenocepacia could be cleared by other phagocytes, we assessed the uptake of apoptotic neutrophils by MDMs using a flow cytometric assay similar to one previously described (22). As a control, we induced apoptosis by cross-linking of neutrophil CD95 (FAS), using a mouse anti-human CD95 Ab with an anti-mouse Ab. Cross-linking of FAS was a potent proapoptotic stimulus and yielded similar levels of apoptosis as challenge with B. cenocepacia (data not shown).

When MDMs (identified by their high CD14 expression) were mixed with apoptotic neutrophils labeled with the cell tracker dye CFSE, a substantial proportion of CD14-positive MDMs acquired CFSE fluorescence, indicating that the MDMs had associated with...
apoptotic neutrophils (Fig. 5A). The actin filament-disrupting agent cytochalasin D largely inhibited the association, demonstrating that most of these interactions represented ingestion of the apoptotic neutrophils by MDMs (Fig. 5A). This was confirmed by fluorescent microscopy of DAPI-stained slides, showing MDMs engulfing one or more CFSE-positive neutrophils (Fig. 5B). The proportion of CD14-positive cells displaying CFSE fluorescence in the presence or absence of cytochalasin D was determined for neutrophils rendered apoptotic either by ingestion of B. cenocepacia or by cross-linking of FAS. Both types of apoptotic neutrophils were ingested to a similar extent by the MDMs (Fig. 5C). Thus, we concluded that neutrophils having phagocytosed viable B. cenocepacia became apoptotic and could be cleared by MDMs.

**Neutrophil ROS production**

Oxidative stress has long been known as an important proapoptotic factor (23), and neutrophils from patients with CGD (which are unable to form ROS) display delayed spontaneous apoptosis in vitro (11). Furthermore, intracellular production of ROS can induce neutrophil apoptosis (7), and ROS scavengers and/or NADPH-oxidase inhibitors prevent apoptosis following phagocytosis of a variety of microbes (8-10, 24, 25). The neutrophil NADPH-oxidase is localized in two cellular pools: in the membrane of intracellular organelles (specific granules), and in the plasma membrane, and neutrophils can produce ROS intracellularly as well as extracellularly (18). Phagocytosis of bacteria is often accompanied by activation of the NADPH-oxidase, resulting in ROS generation inside the phagosome. Robust intracellular ROS production was measured when neutrophils were stimulated with B. cenocepacia, peak after ~60 min and then declining slowly (Fig. 6). A very similar oxidative burst was noted when neutrophils were stimulated with UV-killed bacteria, indicating that bacterial viability did not influence the oxidative response of the phagocytes. That UV-killed B. cenocepacia failed to induce apoptosis, even though the bacterial cells were both phagocytosed and induced ROS production, indicated that ROS generation was not sufficient to induce neutrophil apoptosis. The oxidative burst induced by phagocytosis of bacteria (viable or UV killed) was comparable in magnitude and duration, but differed kinetically, from that induced by PMA and could be inhibited by DPI (Fig. 6).

**Apoptosis or necrosis and the implications of ROS**

The fact that UV-killed B. cenocepacia did not induce neutrophil apoptosis even though it was phagocytosed (Fig. 1B) and induced an oxidative burst (Fig. 6) indicated that the proapoptotic effect of viable bacteria was not exclusively due to the formation of ROS. To further investigate the role of ROS, we performed challenge experiments on neutrophils from CGD patients. Consistent with previous publications (9, 11), levels of spontaneous apoptosis were consistently lower for CGD neutrophils than for control neutrophils (Fig. 7), although not statistically significant. Challenge with B. cenocepacia induced apoptosis also in CGD neutrophils, and the levels of apoptosis, defined as percentage of cells positive for annexin V and negative for EthD, were not suppressed in the absence of DPI (Fig. 7). In fact, when neutrophils were unable to mount an oxidative response (CGD neutrophils or normal neutrophils in the presence of DPI), challenge resulted in slightly higher proportions of apoptotic cells than did challenge of normal neutrophils with the ability to produce ROS unaltered. These differences, however, failed to

![FIGURE 5.](image-url) Apoptotic neutrophils are cleared by MDMs. A, CD14-stained MDMs acquired CFSE fluorescence of apoptotic neutrophils when co-culturing took place in the absence of cytochalasin D. B, Cells were cytospun and DAPI stained to visualize phagocytosis of apoptotic neutrophils (green with condensed blue nucleus) by MDMs (blue nucleus). C, The percentage of MDMs (CD14+) acquiring CFSE fluorescence in the presence or absence of cytochalasin D (5 μg/ml) was determined using flow cytometry. Neutrophils were rendered apoptotic due to challenge with B. cenocepacia or incubation with α-FAS + cross linking Ab (as described in the text) and the uptake quantified using flow cytometry. The figure shows mean ± SEM of three different experiments.

![FIGURE 6.](image-url) B. cenocepacia induces intracellular ROS production in neutrophils. Normal neutrophils were stimulated with viable or UV-killed bacteria (multiplicity of infection 1:30), and the intracellular ROS production was measured continuously by luminol-ECL in the presence of extracellular scavengers superoxide dismutase and catalase. Also shown are control stimulations using PMA (100 ng/ml) or viable bacteria in the presence of DPI (10^{-5} M). A representative experiment is shown.

![FIGURE 7.](image-url) B. cenocepacia induces apoptosis in a ROS-independent manner. Normal neutrophils (in the presence or absence of DPI) or CGD neutrophils were challenged with B. cenocepacia, and apoptosis was assessed by flow cytometry. Cells positive for annexin V and negative for EthD were defined as apoptotic. Data are mean ± SEM from three to seven independent experiments.
reach statistical significance. These results indicated that the induction of apoptosis occurred in a ROS-independent manner.

More dramatically, when neutrophils from CGD patients were challenged with *B. cenocepacia*, a significant proportion of cells exhibited EthD fluorescence, indicative of a leaky plasma membrane (Fig. 8 and Table I). This suggested that CGD neutrophils became necrotic after *B. cenocepacia* challenge, and similar results were obtained using normal neutrophils in the presence of DPI (Table I). Necrotic cell death was also evaluated by measuring the leakage of cellular LDH after challenge. These analyses clearly showed that substantial necrosis occurred only when neutrophils incapable of producing ROS were challenged with viable bacteria (Fig. 8 and Table I). Taken together, these data suggest that ROS are not involved in promoting neutrophil apoptosis following phagocytosis of *B. cenocepacia*, but are critical for detoxifying ingested bacteria to abrogate necrosis of the ingesting neutrophil.

**Discussion**

Although the inflammatory reaction is crucial for host defense against infection, this process is a double-edged sword that when persistent may cause a number of complications and even facilitate bacterial spread. Neutrophils are short-lived, potent inflammatory cells that are normally cleared by undergoing apoptosis and subsequently being ingested by macrophages, processes that are critical for resolution of inflammation (13). The uptake of apoptotic neutrophils facilitates noninflammatory removal of the phagolysosomal mediators and proteolytic enzymes residing within these cells, and at the same time actively suppresses secretion of inflammatory mediators from the ingesting macrophages (26). Neutrophils that die by necrosis, instead of apoptosis, release their toxic contents in an uncontrolled fashion, and macrophages ingesting necrotic cells may become activated to secrete high levels of proinflammatory cytokines (27). Thus, apoptosis and clearance are doubly beneficial in the termination of an inflammatory response, but necrosis may have profound toxic implications on the outcome of an infection.

The molecular mechanisms underlying neutrophil apoptosis have been extensively studied (reviewed by Simon (23) and Akgul et al. (12)), and the process can be either accelerated or delayed by death and survival signals, respectively. Upon phagocytosis of a microbial prey, in most cases the neutrophils activate its apoptotic program (28). However, some pathogens, such as *Streptococcus pyogenes* (28) and *Chlamydia pneumoniae* (29), modulate the neutrophil response to inhibit apoptosis. In both cases, the neutrophils fail to kill the ingested bacteria, and the delay of apoptosis may be a bacterial strategy for establishing intracellular parasitism. In general, ROS appear to be important proapoptotic mediators, but the exact nature of their interaction with other death molecules, e.g., caspases, has not been determined (12, 23). Regardless of how ROS affect apoptotic processes, studies on neutrophils from CGD patients have shown that these cells display a slower rate of spontaneous apoptosis compared with neutrophils from normal donors (9, 11). CGD neutrophils also fail to induce the proapoptotic regulator BAX upon phagocytosis (30), and this could be the reason for the apoptotic defects displayed after the binding of death receptors (11) and phagocytosis of IgG- and C3bi-coated latex beads (30).

Our data demonstrated that normal neutrophils produced substantial amounts of ROS during phagocytosis of *B. cenocepacia*, and subsequently became apoptotic. Using both neutrophils from CGD patients and normal neutrophils in the presence of DPI, we next investigated whether the ROS production was directly linked to the induction of apoptosis. We found that apoptosis after bacterial challenge was not reduced in the absence of ROS. Moreover, only viable *B. cenocepacia* promoted apoptosis, despite the fact that UV-killed and live bacteria were ingested and induced ROS production at comparable levels. Taken together, these data demonstrate that ROS production was neither sufficient, nor necessary.

**Table 1. The absence of ROS promotes necrotic cell death after *B. cenocepacia* challenge**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (n = 7)</th>
<th>Control + DPI (n = 6)</th>
<th>CGD (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cenocepacia</em> (LDH release)</td>
<td>3.8 ± 1.7</td>
<td>5.3 ± 1.6</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>Spontaneous (annexin V$^+$/EthD$^+$)</td>
<td>7.7 ± 2.0</td>
<td>18.9 ± 2.5$^b$</td>
<td>20.2 ± 3.3$^b$</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> (annexin V$^+$/EthD$^+$)</td>
<td>10.7 ± 2.2</td>
<td>11.3 ± 1.7</td>
<td>8.5 ± 2.4</td>
</tr>
<tr>
<td>Necrotic Neutrophils (% of total ± SD)$^a$</td>
<td>24.9 ± 5.9</td>
<td>40.4 ± 5.2</td>
<td>56.4 ± 16.2$^b$</td>
</tr>
</tbody>
</table>

$^a$ Neutrophils were cultured without treatment (Spontaneous) or after challenge with viable *B. cenocepacia*, and necrosis was quantified either by flow cytometry (necrotic cells were defined as annexin V$^+$/EthD$^+$) or by measuring release of cellular LDH to the supernatants.

$^b$ Significantly different (p < 0.05) from control cells receiving the same treatment and analyzed using the same method.
for inducing apoptosis. This is in contrast to most earlier studies that, using DPI or different antioxidants, report that in the absence of ROS the proapoptotic effects induced by the phagocytosis of microbes are inhibited (8–10, 25). Although chemicals such as DPI have been used to mimic the conditions in CGD (31), they may affect cells in other ways than by simply preventing ROS formation (32). Neutrophils from CGD patients have been used to demonstrate that ROS-dependent pathways are, at least partially, responsible for induction of neutrophil apoptosis following ingestion of heat-killed S. aureus (9). Thus, there appear to exist both ROS-dependent and ROS-independent mechanisms for inducing neutrophil apoptosis.

A striking and clinically relevant difference between normal and CGD neutrophils challenged with B. cenocepacia was the induction of necrotic cell death in CGD neutrophils. After bacterial challenge, apoptotic cell death was induced in both normal and CGD neutrophils; only in CGD cells, however, could necrosis be detected. We measured necrosis using two different techniques, cellular uptake of the nuclear binding dye EthD and leakage of cellular LDH; both techniques showed significantly more necrosis when the challenged cells were unable to produce ROS (Table I). The exact molecular mechanisms by which the different types of cell death are mediated and where ROS fit into these processes are largely unknown. It is possible that the different fate of CGD neutrophils after bacterial challenge is due to an altered degranulation pattern and impaired phagolysosomal fusion compared with normal neutrophils. Another intriguing possibility is that ROS somehow influence the activity of different types of caspases that may dictate the outcome. Regardless of the underlying molecular determinants, our data clearly show that neutrophils unable to produce ROS become necrotic after challenge with B. cenocepacia. Taken together with the remarkable resistance to nonoxidative means of neutrophil killing displayed by this group of bacteria (5), it may be that neutrophils from CGD patients, challenged with a bacterium resistant to nonoxidative killing, are more prone to undergo necrotic cell death simply because the engulfed prey is not properly killed.

Our data could explain why B. cenocepacia is often such a problematic pathogen in CGD patients, while only rarely, if ever, causing any problems in normal individuals. Healthy individuals are able to effectively cope with a B. cenocepacia infection, and their neutrophils become apoptotic after ingestion of the bacteria. These apoptotic neutrophils can then be cleared by macrophages in an anti-inflammatory manner, ensuring a proper termination of the inflammatory process. In CGD patients, however, infection with B. cenocepacia could be highly destructive if the ingesting neutrophils failed to kill the bacteria and then became necrotic. Instead of resolving the inflammation, such a neutrophil-bacterial interaction would promote tissue destruction and a persistent inflammatory process. The resulting tissue damage could also provide a means for bacteriemic spread of this organism, a pathological feature that is rare with other bacterial species in CGD patients (4).

B. cenocepacia is also a very problematic pathogen in patients with CF. Unlike the more common CF pathogen, P. aeruginosa, B. cenocepacia can cause necrotizing pneumonia and life-threatening septicemia. Numerous reports suggest that an oxidant/antioxidant imbalance exists in the lungs of CF patients, even though CF neutrophils are not impaired in ROS production in vitro; molecules found in CF secretions (e.g., bacterial mucoid exopolysaccharide and host mucin) can quench ROS (33–35). It is possible that such quenching would protect B. cenocepacia complex bacteria from ROS-mediated killing, creating a situation akin to CGD with necrotic cell death and persistent destructive inflammation.

In summary, we have demonstrated that B. cepacia complex bacteria have the innate capacity to cause devastating necrosis under conditions in which ROS production is compromised. Patients with two dissimilar diseases, CGD and CF, may be particularly susceptible to this opportunistic pathogen because either ROS are not produced (CGD) or their capacity to kill bacteria is abrogated due to redox imbalance at the site of infection (CF). In both diseases, novel interventions might be designed by taking advantage of these observations.

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**References**


