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Functional Activity of Individual Abscess Neutrophils from Mice

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In the absence of antibiotic therapy, viable bacteria can persist within intra-abdominal abscesses in mice for at least 10 weeks. The mechanisms contributing to this survival are unknown, but abscess-derived neutrophils have impaired abilities to kill, in vitro, organisms engulfed in vivo. In order to determine whether subpopulations of abscess neutrophils might be discernible on the basis of phenotypic or functional criteria, cells from murine intra-abdominal abscesses were examined for phagocytic activity, CR3 expression, and H_2O_2 production in response to soluble and particulate stimuli. With respect to phagocytosis of *Proteus mirabilis*, abscess cells were no less efficient than peritoneal exudate neutrophils; no significant subpopulation of cells was incapable of phagocytosis in the presence of normal mouse serum. Using flow cytometry to examine abscess neutrophils for CR3 expression, we found that no subpopulations of cells were observed with unstimulated cells or with cells incubated with either phorbol 12-myristate 13-acetate or bacteria and serum. Intracellular H_2O_2 levels were measured by using the probe 2′,7′-dichlorofluorescin diacetate. In general, incubation with phorbol 12-myristate 13-acetate resulted in similar increases in H_2O_2 production in all cells of the population. However, stimulation with bacteria and serum revealed a variable but consistent, poorly responsive subpopulation of neutrophils in abscess cell populations. Cell-sorting experiments showed that cells from the poorly responsive section of the FACS profile contained significantly higher numbers of abscess-derived bacteria, suggesting the presence of a subpopulation of viable abscess neutrophils harboring persisting viable bacteria.

Intra-abdominal abscesses can be induced experimentally in mice by intraperitoneal inoculation of an abscess-potentiating agent, such as bran, together with any of a range of bacterial species, either alone or in combination. Depending on the species used for induction, abscesses taken from a single animal 7 days after inoculation may contain 10^7 to 10^8 viable bacteria. By using mechanical disaggregation and Percoll density gradient enrichment, it is possible to obtain 10^6 to 10^7 viable neutrophils from these abscesses. While most of the bacteria in abscesses appear therefore to be extracellular, it is possible that many bacteria in dead and dying neutrophils are released during the preparative procedures. Nevertheless, up to 20% of the neutrophils obtained from abscesses may contain intracellular bacteria. Isolation of both of these populations of abscess bacteria has shown that both intracellular and extracellular organisms are susceptible to phagocytosis and killing in vitro in the presence of normal mouse serum (NMS) and peritoneal exudate neutrophils (J. J. Finlay-Jones, P. H. Hart, L. K. Spencer, M. F. Nulsen, P. A. Kenny, and P. J. McDonald, J. Med. Microbiol., in press). Furthermore, the neutrophils isolated from an abscess efficiently phagocytose and kill bacteria to which they are exposed in vitro in the presence of NMS. However, under the same conditions, intracellular bacteria phagocytosed within the abscess are killed significantly less efficiently, particularly in the case of gram-negative bacteria (5).

However, such in vitro bactericidal assays do not permit individual neutrophils to be assessed functionally, and it is possible that bacteria engulfed in vivo are contained within a subpopulation of viable but inactive neutrophils and act as a source of persisting infection. Microscopic examination of abscess neutrophils after an in vitro phagocytic assay is one means of observing the function of individual abscess neutrophils. A second means involves the use of fluorescent probes and flow cytometry. For example, it has been shown that murine abscess-derived neutrophils express relatively uniform levels of both complement receptor CR3 and Fc receptor FcγRII (Finlay-Jones et al., in press) when fluorescent antibodies and flow cytometry are used. Similarly, the membrane potential (8), intracellular pH (10), calcium level (11), and products of the neutrophil oxidative burst (2) of individual cells can be assessed by using flow cytometry. In the case of products of the neutrophil oxidative burst, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is taken up by neutrophils and subsequently hydrolyzed to nonfluorescent 2′,7′-dichlorofluorescin. Stimulation of a respiratory burst within the cells results in oxidation of 2′,7′-dichlorofluorescin to fluorescent dichlorofluorescein (DCF). This rapid intracellular oxidation is thought to be mediated by H_2O_2, but also appears to require the activity of intracellular peroxidases.

By using several of the techniques described above, individual abscess neutrophils, as well as peritoneal exudate and bone marrow neutrophils, were assessed for phagocytic activity, cell surface receptor expression, and respiratory burst activity in response to soluble and particulate stimuli.

MATERIALS AND METHODS

Mice. Male BALB/c mice that were 4 to 8 weeks old were obtained from the Department of Agriculture, South Australia, Australia. The ethical guidelines of the National Health and Medical Research Council, the Commonwealth Scientific and Industrial Research Organisation of Australia, and the Australian Agricultural Council were followed in all experiments.

Reagents. PMA (catalog no. P8139; Sigma Chemical Co., St. Louis, Mo.) was stored as a 1-mg/ml solution in dimethyl sulfoxide at −80°C. *N*-Formyl-methionyl-leucyl-phenylalanine (N-FMLP) (catalog no. F-3506; Sigma) was stored as a 10^{-2} M solution in dimethyl sulfoxide at −80°C. Recombi-

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nant murine tumor necrosis factor alpha (a gift from Genentech, Inc., San Francisco, Calif.) was stored as a 0.98-mg/ml solution at 4°C. The recombinant murine tumor necrosis factor alpha was titrated for activity in the L929 cytotoxicity assay (7). DCFH-DA (Eastman Kodak, Rochester, N.Y.) was prepared as a 500 μM solution in ethanol and stored at −20°C. Mouse osmolality phosphate-buffered saline (MPBS) was prepared as described by Sheridan and Finlay-Jones (9).

Bacteria. The origins and preparation of frozen stock suspensions of *Escherichia coli*, *Proteus mirabilis*, *Bacteroides fragilis*, and *Staphylococcus aureus* were as previously described (5). For use in in vitro assays, small aliquots of thawed suspensions were inoculated into Trypticase soy broth and incubated at 37°C for 3 to 4 h, and the log-phase bacteria were washed three times in 0.9% saline before resuspension at the required concentration in MPBS.

NMS. Mice were anesthetized with fluorothane (ICI Pharmaceuticals, Macclesfield, United Kingdom) and bled from the heart with a 21-gauge needle. Blood was allowed to clot and was stored on ice for 2 h. The blood was then overlaid with Serasieve (Hughes and Hughes Ltd., Essex, United Kingdom) and centrifuged at 1,000 × *g* for 10 min, and the supernatant serum was filter sterilized and stored in aliquots at −81°C.

Opsonization of bacteria. Bacteria were suspended in NMS (10^9 bacteria in 1.0 ml of NMS), and each suspension was mixed with an equal volume of RPMI 1640 medium (pH 7.2), supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) at 37°C for 30 min. The bacteria were subsequently washed twice with 0.9% saline prior to resuspension in the appropriate buffer.

Intra-abdominal abscess induction. As described previously (5), abscesses were induced by intraperitoneal injection of 1 mg of bran together with either a single bacterial species or two bacterial species; *B. fragilis* (5×10^8 CFU) was injected together with *E. coli* (5×10^6 CFU), or *E. coli*, *P. mirabilis*, or *S. aureus* was injected alone (10^7 CFU). Each abscess-inducing mixture was diluted in RPMI 1640 medium (pH 7.2) (Flow Laboratories Inc., McLean, Va.) buffered with 10 mM HEPES. Each mouse was injected with 50 μl of abscess-inducing mixture intraperitoneally, and abscesses were harvested 6 to 12 days after injection. Generally, abscesses harvested from groups of 15 to 30 mice were employed for individual experiments. Each figure shows the results obtained from separate groups of animals.

Isolation of leukocytes from abscesses and peritoneal exudates. The preparation of single-cell suspensions of leukocytes from intra-abdominal abscesses and peritoneal exudates and the fractionation of neutrophil-enriched populations by Percoll density gradient centrifugation have been described previously (4). However, abscess disaggregation was carried out at 4°C in order to reduce nonspecific neutrophil activation. The percentages of the 100% Percoll stock solution used for fractionation of abscess cells were 70, 45, and 35% (densities, 1.0871, 1.0575, and 1.0456 g/ml, respectively), and the percentages of the 100% Percoll stock solution used for fractionation of peritoneal exudate cells were 81, 65, and 55% (densities, 1.1002, 1.0812, and 1.0693 g/ml, respectively). Generally, cells isolated from the interface between the two most dense layers of Percoll were used.

Isolation of leukocytes from fresh and cultured mouse bone marrow. Femoral and tibial bone marrow samples were collected into cold culture medium (3) by washing out with a 26-gauge needle, and single-cell suspensions were prepared by flushing the wash-out through a 19-gauge needle. The cells were then washed once (200 × *g*, 10 min) in culture fluid. Approximately 10^8 cells were then distributed in 20-ml volumes into 260-ml tissue culture flasks (Nunc) for incubation at 37°C in a humidified atmosphere containing 10% CO_2 in air. For cell culture, cells were maintained in RPMI 1640 medium containing 15% (vol/vol) fetal calf serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM pyruvate, 7.5×10^{-5} M monothioglycerol, 10^{-6} M hydrocortisone 21-hemisuccinate, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. A 10-ml portion of culture fluid containing nonadherent cells was removed each week and replaced with 10 ml of fresh medium. For use, fresh bone marrow wash-outs or culture-derived cells were washed in MPBS and resuspended in Hanks balanced salts solution buffered with 10 mM HEPES (pH 7.35) at a concentration of 2.5×10^6 cells per ml. Suspensions were fractionated on Percoll density gradients by using concentrations of the 100% Percoll stock solution of 81, 65, and 55% (densities, 1.1002, 1.0812, and 1.0693 g/ml, respectively). The neutrophil-enriched fractions were washed three times (200 × *g*, 10 min) prior to resuspension at the appropriate concentration in MPBS or RPMI 1640 medium.

Monoclonal antibodies. Monoclonal antibody NIMP-R10, which recognizes mouse complement receptor CR3 (6), is a rat immunoglobulin G2b molecule and was kindly donated by Angel Lopez (Institute of Medical and Veterinary Science, Adelaide, Australia). Serum and ascitic fluid from hybridoma-inoculated rats and culture supernatants were dialyzed against MPBS overnight prior to dilution in the appropriate buffer and centrifuged at 8,000 × *g* for 15 min to remove aggregates.

Flow cytometric analysis of receptor expression. A total of 10^6 cells were pelleted in a serological tube, and the pellet was resuspended in 100 μl of the appropriate monoclonal antibody or MPBS. The suspensions were incubated on ice for 20 min and resuspended to a volume of 3 ml in MPBS containing 0.02% (wt/vol) NaN_3, and the cells were pelleted by centrifugation at 200 × *g* for 10 min. The pellets were resuspended in 100 μl of MPBS containing 0.02% (wt/vol) NaN_3 and an optimal concentration of fluorescein isothiocyanate-labeled goat anti-rat immunoglobulin G (catalog no. F-6258; Sigma), and the tubes were incubated on ice for 20 min. The cells were subsequently washed twice in MPBS containing 0.02% (wt/vol) NaN_3 and finally resuspended in 200 μl of fixative (1% formaldehyde, 2% [wt/vol] glucose, and 5 mM NaN_3 in MPBS). Fluorescence profiles of these cells were obtained by using a Becton Dickinson model FACS analyzer (Becton Dickinson, Mountain View, Calif.) equipped with a mercury arc lamp that provided excitation at 485 ± 11 nm. Fluorescent emission at 530 ± 15 nm was recorded, and the voltage of the logarithmic amplifier was adjusted to maintain fluorescence intensities within a 1,000-channel range. Neutrophils were gated on the basis of volume and 90° light scatter.

Flow cytometric measurement of respiratory burst activity of neutrophils. A suspension containing 10^7 cells per ml in MPBS was incubated with 5 μM (final concentration) DCFH-DA at 37°C in a shaking water bath for 15 min. Generally, cold MPBS was then added, and the cells were washed once (200 × *g*, 10 min) and finally resuspended at a concentration of 10^7 cells per ml in MPBS. Aliquots (100 μl) were then added to 100 μl of MPBS containing the appropriate stimuli, and the tubes were incubated at 37°C in a shaking water bath for 15 to 30 min. In some instances cells were not washed after incubation with DCFH-DA but were washed after incubation with the stimuli. Also, in later

TABLE 1. Phagocytosis of *P. mirabilis* by abscess neutrophils and peritoneal exudate neutrophils

Neutrophil source	Bacterium-to-cell ratio	% Of neutrophils with leukocyte-associated bacteria[a]	% Of neutrophils with >9 leukocyte-associated bacteria[a]
Abscess[b]	50:1	98 ± 4	96 ± 4
	10:1	91 ± 3	39 ± 1
	2:1	37 ± 16	0 ± 0
Peritoneal exudate	50:1	100 ± 0	98 ± 2
	10:1	89 ± 6	5 ± 4
	2:1	11 ± 2	0

[a] Mean ± standard deviation of triplicate values.
[b] Abscesses were induced with an abscess-inducing mixture consisting of bran (1 mg), *B. fragilis* (5×10^8 CFU), and *E. coli* (3×10^6 CFU). The percentage of abscess neutrophils containing abscess-derived bacteria was 10%.

experiments, incubations were performed in microtiter plates (Disposable Products, Adelaide, Australia) shaken at 1300 rpm on an orbital plate shaker (Titertek; Flow Laboratories). Fluorescence profiles were obtained for individual samples as described above for cell surface receptor analysis.

Fluorescence-activated cell sorting. Samples of unstimulated and stimulated DCF-labeled neutrophil-enriched populations prepared as described above were fractionated on the basis of fluorescence intensity by using a model FACS IV cell sorter (Becton Dickinson); a laser power of 500 mW at 488 nm provided excitation. Fluorescent emission at 530 ± 15 nm was recorded, and the voltage of the logarithmic amplifier was adjusted to maintain fluorescence intensities within a 10,000-channel range. Sorted cell populations were counted and subsequently diluted in 0.9% (wt/vol) NaCl

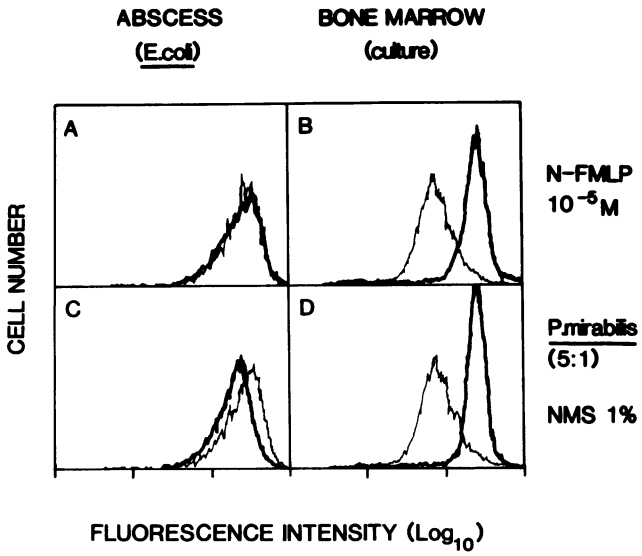

FIG. 1. Cell surface expression of CR3 on stimulated and unstimulated mouse neutrophils. Fractionated abscess (*E. coli*-induced) and bone marrow neutrophils were treated with either MPBS (light lines) or stimuli (heavy lines) for 30 min at 37°C with shaking. (A and B) Stimulation with 10^{-5} M N-FMLP. In the case of stimulation by *P. mirabilis* and 1% NMS (C and D), the bacterium-to-cell ratio was 5:1. The cells were labeled with monoclonal antibody NIMP-R10 and then with fluorescein isothiocyanate-labeled goat anti-rat immunoglobulin G, and then they were fixed and analyzed for receptor expression by flow cytometry.

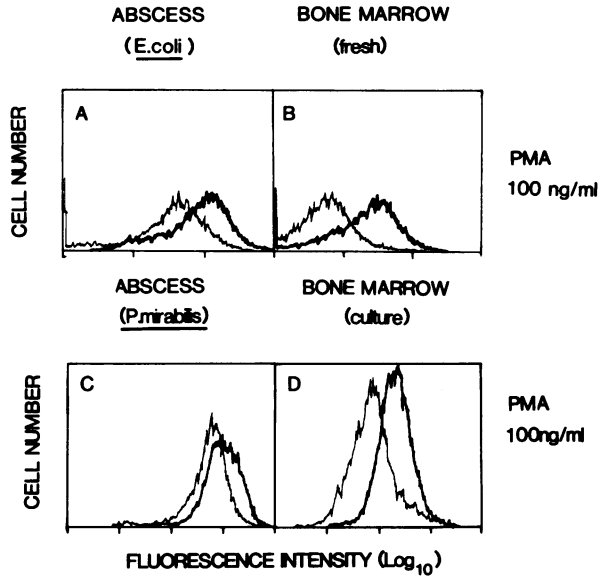

FIG. 2. Fluorescence profiles of DCF-labeled mouse neutrophils stimulated with PMA. Fractionated abscess (*E. coli*-induced [A] or *P. mirabilis*-induced [C]) and bone marrow neutrophils (B and D) were incubated with DCFH-DA, and the washed cells were treated with either MPBS (light lines) or PMA (100 ng/ml) (heavy lines) for 15 to 30 min at 37°C with shaking. The cells were washed again and used directly for analysis of intracellular DCF production by flow cytometry.

containing 0.05% (vol/vol) Triton X-100 to release intracellular bacteria. Dilutions of the lysate were then plated onto MacConkey and horse blood agar media for culture at 37°C aerobically and anaerobically, respectively.

Phagocytic assay. Aliquots (80 μl) of RPMI 1640 medium (Flow Laboratories) supplemented with 10 mM HEPES (pH 7.2) and containing 5×10^7 or 10^7 CFU of washed *P. mirabilis* were added to flat-bottom microtiter plates (catalog no. 23149; Disposable Products). Aliquots (100 μl) containing 10^6 abscess or peritoneal exudate neutrophils were added, and the volume in all wells was adjusted to 200 μl with either 20 μl of RPMI 1640 medium or 20 μl of 10% (vol/vol) NMS. The plates were incubated at 37°C for 30 min on an orbital plate shaker (1,300 rpm), and after incubation samples were removed for preparation of cytocentrifuge smears. Smears were stained with Jenner-Giemsa reagents for examination by light microscopy.

RESULTS

The ability of Percoll-fractionated abscess neutrophils to phagocytose *P. mirabilis* in the presence of 1% NMS was compared with the phagocytic activity of peritoneal exudate neutrophils. First, it was found that at a bacterium-to-neutrophil ratio of 50:1, 96% of the abscess neutrophils had more than nine leukocyte-associated bacteria; this included many of the cells that already contained abscess-derived bacteria (Table 1). Second, at each of the ratios of bacteria to cells tested, abscess neutrophils were at least as efficient as peritoneal exudate neutrophils in terms of uptake of *P. mirabilis* (Table 1).

Attempts were made to identify subpopulations of abscess neutrophils on the basis of expression of the complement receptor CR3. As has been found previously (Finlay-Jones et al., in press), unstimulated abscess neutrophils expressed a

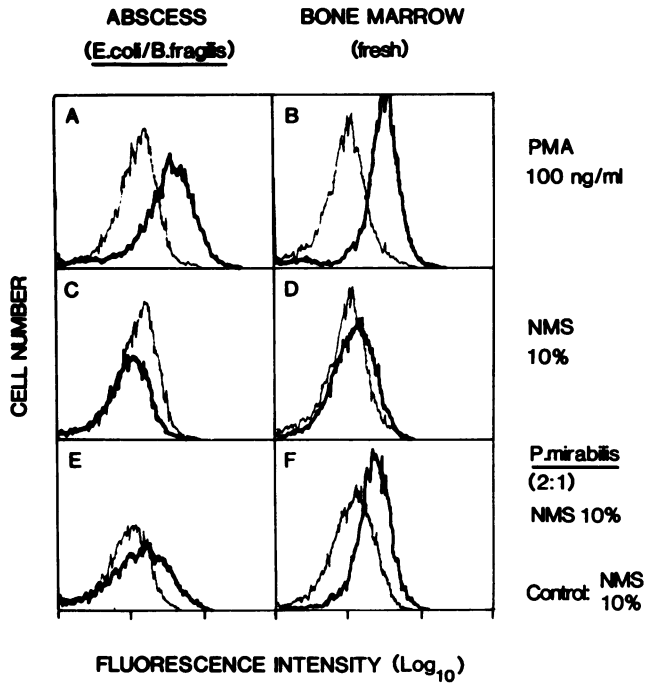

FIG. 3. Fluorescence profiles of DCF-labeled mouse neutrophils. Fractionated abscess (*E. coli–B. fragilis*-induced) and bone marrow-derived neutrophils were incubated with DCFH-DA, and the washed cells were treated with either MPBS or stimuli for 15 to 30 min at 37°C with shaking. The cells were washed again and used directly for analysis of intracellular DCF production by flow cytometry. (A through D) MPBS control (light lines) and cells treated with 100 ng of PMA per ml (A and B) or NMS (C and D) (heavy lines). (E and F) Control profile (light lines) is the profile for cells treated with NMS only, and the stimulated cells (heavy lines) were treated with *P. mirabilis* (bacterium-to-cell ratio, 2:1) and 10% NMS.

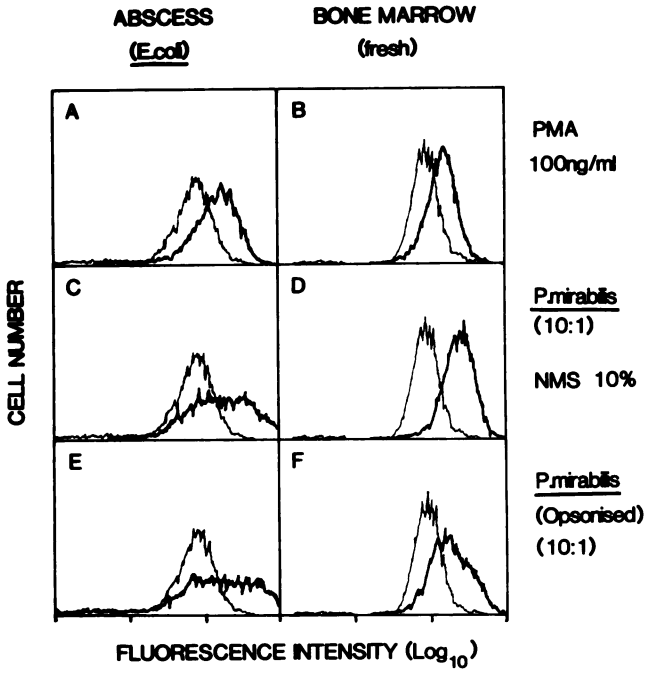

FIG. 4. Fluorescence profiles of DCF-labeled abscess and bone marrow neutrophils. Fractionated abscess (*E. coli*-induced) and bone marrow neutrophils were incubated with DCFH-DA, the washed cells were treated with MPBS (light lines) or stimuli (heavy lines), and the cells were analyzed for intracellular DCF production by flow cytometry. (A and B) Stimulation with 100 ng of PMA per ml. (C and D) Stimulation with *P. mirabilis* (bacterium-to-cell ratio, 10:1) and 10% NMS. (E and F) Stimulation with opsonized *P. mirabilis* (bacterium-to-cell ratio, 10:1).

uniform level of CR3, as detected by using monoclonal antibody NIMP-R10, and this was also the case for bone marrow neutrophils (Fig. 1). As expected, bone marrow neutrophils responded to stimulation with either N-FMLP or *P. mirabilis* and 1% NMS with significant and uniform upregulation of CR3 (Fig. 1). Abscess neutrophils which when unstimulated exhibited a high level of CR3 (mean fluorescence intensity of 275 versus 91 for unstimulated bone marrow neutrophils) did not exhibit upregulation of CR3 after stimulation with N-FMLP and showed a uniform decrease in the level of fluorescence after stimulation with bacteria and serum (Fig. 1). Consequently, it was not possible to identify subpopulations of abscess neutrophils on the basis of CR3 expression. This was also the case for abscess neutrophils derived from *S. aureus*-induced abscesses (data not shown).

We then examined abscess-derived neutrophils for H_2O_2 production in response to treatment with PMA by using the intracellular fluorescent probe DCF and flow cytometry. It appeared that all viable neutrophils from an abscess, regardless of the inducing organism, were capable of exhibiting a respiratory burst, as indicated by the increase in fluorescence intensity shown by all cells in the abscess cell population (Fig. 2). This was also the case for control populations of bone marrow neutrophils (Fig. 2). The pattern of response observed after PMA stimulation of abscess cells (i.e., a unimodal shift in the fluorescence intensity distribution) was observed regardless of the organisms used for abscess induction (Fig. 2A and C, 3A, 4A, 5A, and 6A). It was then important to determine whether a similar pattern of respiratory burst activity occurred after stimulation of abscess neutrophils through complement receptor CR3. It had pre-

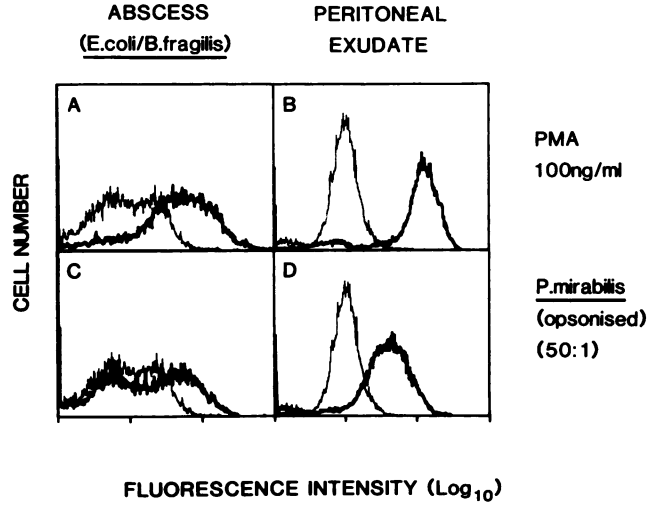

FIG. 5. Fluorescence profiles of DCF-labeled mouse neutrophils. Fractionated abscess (*E. coli–B. fragilis*-induced) and peritoneal exudate neutrophils were incubated with DCFH-DA, and the washed cells were treated with MPBS as controls (light lines) or stimulated (heavy lines) with 100 ng of PMA per ml (A and B) or opsonized bacteria (C and D) (bacterium-to-cell ratio, 50:1).

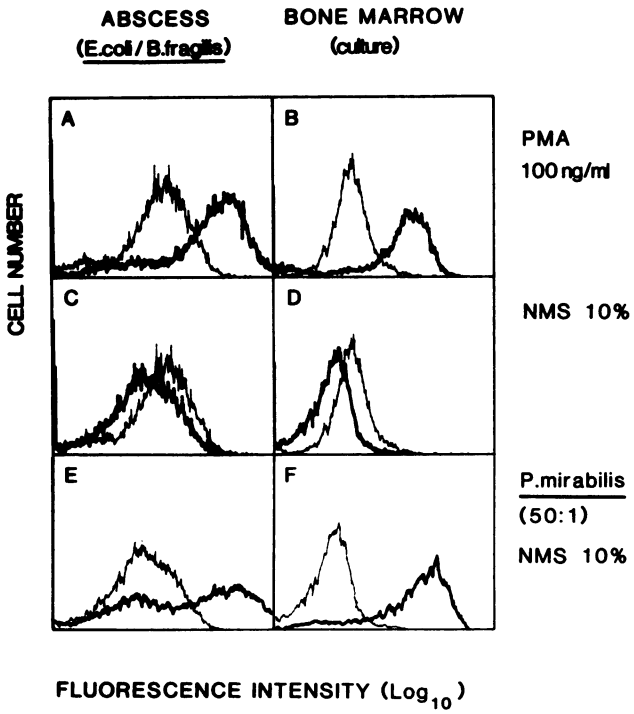

FIG. 6. Fluorescence profiles of DCF-labeled mouse neutrophils. Fractionated abscess (*E. coli–B. fragilis*-induced) and bone marrow neutrophils were incubated with DCFH-DA, and the washed cells were treated with MPBS, PMA, NMS, or bacteria and NMS for 15 to 30 min at 37°C with shaking. The cells were then analyzed for intracellular DCF production by flow cytometry. (A through D) The controls (light lines) were cells treated with MPBS, and the stimulated cells (heavy lines) were treated with 100 ng of PMA per ml (A and B) or 10% NMS (C and D). (E and F) NMS-treated cells were the controls (light lines); other cells were treated with *P. mirabilis* (bacterium-to-cell ratio, 50:1) and 10% NMS (heavy lines).

viously been shown that blocking of this receptor with monoclonal antibody significantly reduced both the phagocytosis and the killing of bacteria by a range of neutrophil types (4). Figure 3 shows a comparison between abscess neutrophils (induced by *E. coli* and *B. fragilis*) and bone marrow neutrophils with respect to their fluorescence profiles after incubation in buffer only, in PMA, in NMS, or in *P. mirabilis* and NMS. Once again, we found that PMA induced a similar increase in fluorescence in all cells of both cell populations. In contrast, stimulation with *P. mirabilis* (bacterium-to-cell ratio, 2:1) and NMS induced a range of levels of H_2O_2 production in abscess cells, as indicated by the broadened fluorescence profile, but much more uniform shifts in fluorescence in control bone marrow cells (Fig. 3). A similar effect occurred with *E. coli*-induced abscesses (that is, a broad range of respiratory burst responses in response to stimulation with *P. mirabilis* and NMS) (Fig. 4).

The shape of the fluorescence profile in the DCF assay with abscess cells was variable, as was the increase in the mean fluorescence intensity observed after stimulation with either PMA or opsonized bacteria. In addition to the profile patterns shown in Fig. 3 and 4, in some instances two populations of abscess cells were observed in fluorescence profiles of unstimulated cells (Fig. 5 and 6). In such cases, stimulation with PMA induced a respiratory burst in most cells of both abscess cell and bone marrow cell populations. After stimulation with opsonized bacteria or with bacteria

TABLE 2. Bacterial contents of DCF-labeled abscess-derived neutrophils fractionated by flow cytometry

P. mirabilis-cell ratio[a]	% Of cells sorted from low-fluorescence fraction/% of cells sorted from high-fluorescence fraction[b]	Ratio of bacteria/cell for low-fluorescence cells to bacteria/cell for high-fluorescence cells[c]		
		P. mirabilis	*E. coli*	*B. fragilis*
10:1	50/50	0.9	ND[d]	ND
50:1	50/50	0.9	ND	ND
10:1	50/50	1.0	2.2	ND
50:1	50/50	0.7	10.8	ND
50:1	9/7	3.5	3.2	ND
50:1	25/25	1.3	2.5	ND
10:1[e]	13/12	3.1	9.0	6.8

[a] Abscesses were induced by using bran, *B. fragilis*, and *E. coli*. Abscess-derived neutrophils were stimulated with *P. mirabilis* and 10% NMS.

[b] The fraction of cells collected was always the fraction at either extreme (low fluorescence or high fluorescence) of the fluorescence profile.

[c] For the ratio for *E. coli* versus the ratio for *P. mirabilis*, $P = 0.042$ (Mann-Whitney test).

[d] ND, Not determined.

[e] Cells were pretreated with 100 ng of tumor necrosis factor alpha per ml for 30 min at 37°C prior to addition of bacteria and serum.

and NMS, the percentage of responding cells was lower for abscess cells, with those cells showing a higher background fluorescence responding more strongly.

On the basis of the results described above we hypothesized that those abscess cells which responded suboptimally to stimulation through complement receptors contained persisting bacteria which had been engulfed within the abscess. To test this hypothesis, DCFH-DA-labeled abscess cells (induced by *E. coli* and *B. fragilis*) were stimulated with *P. mirabilis* in the presence of NMS, and the fluorescence profiles were examined by using the model FACS IV cell sorter. Fractions of cells from both extremes of the fluorescence profile were sorted, and aliquots of collected cells were lysed with 0.05% Triton X-100. The lysates were spread onto MacConkey and horse blood agar plates for aerobic and anaerobic incubation, respectively. Table 2 shows the ratios of the values for bacteria per cell from the low- and high-fluorescence fractions. In the case of *E. coli*, this ratio varied between 2 and 10 in a series of experiments. In the single experiment in which *B. fragilis* counts were determined, the ratio was 6.8. Analysis of the data for *E. coli* (the organism engulfed within the abscess) and *P. mirabilis* (the organism to which the neutrophils were exposed in vitro) with the Mann-Whitney test showed that the ratio was significantly higher in the case of the *E. coli*-containing cells.

DISCUSSION

It has been established previously that a proportion of neutrophils derived from intra-abdominal abscesses in mice contain bacteria which are significantly less susceptible to killing than fresh organisms added to the abscess cells in vitro (5). However, it has not been possible to isolate the abscess-derived neutrophils containing the intracellular bacteria in order to assess their functional capacity. In this report we describe a series of experiments designed to identify subpopulations of abscess neutrophils which may or may not harbor the persisting intracellular organisms. In terms of complement-dependent phagocytic activity, abscess neutrophils were no less efficient than peritoneal exudate neutrophils. In particular, at the maximum ratio of bacteria to cells, 96% of the neutrophils were associated with more than nine bacteria per cell, as determined microscop-

ically. Therefore, almost all abscess neutrophils are capable of efficient phagocytosis; this includes at least the majority of the cells which already contained organisms engulfed in vivo.

Similarly, it was not possible to identify subpopulations of abscess neutrophils on the basis of CR3 expression. This was because all cells in the population expressed maximal levels of the receptor, such that under appropriate stimulation, the receptor upregulation which is observed with all bone marrow cells (and to a lesser extent with peritoneal exudate cells) did not occur. The connection between CR3 upregulation on neutrophils and antibacterial activities remains unclear. When we used murine neutrophils that were obtained from a range of sites and were stimulated with N-FMLP, PMA, and cytokines, such as tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor (GM-CSF), it was not possible to enhance the bactericidal activity of the neutrophils even though CR3 upregulation could be induced (unpublished data). In the case of human neutrophils, it has been shown that stimulation with PMA can lead to both upregulation of CR3 and activation of the receptors (12). However, extended incubation with PMA resulted in subsequent deactivation of the receptors. This suggests that there are conditions under which neutrophils can express adequate levels of CR3 but that these molecules may not be fully functional. While the ability of abscess neutrophils to phagocytose *P. mirabilis* efficiently argues against the hypothesis that a proportion of such cells exhibit deactivated complement receptors, it is possible that stimulation of the receptors for bactericidal activity, in contrast to phagocytosis, is suboptimal for a proportion of abscess cells. We found that bacteria phagocytosed by peritoneal exudate neutrophils preincubated with PMA for 30 min were killed significantly less efficiently in subsequent in vitro assays than bacteria in untreated cells. The components within the abscess environment which might lead to such selective deactivation of bactericidal activity as distinct from phagocytic activity is unknown.

However, assessment of the respiratory burst of abscess neutrophils via flow cytometry did provide evidence of functional heterogeneity of the abscess cells. While PMA stimulated all abscess cells (induced with a range of bacterial species) to produce H_2O_2 to similar extents, as was the case for control populations of bone marrow and peritoneal exudate neutrophils, incubation with opsonized bacteria or bacteria and NMS at bacterium-to-cell ratios which ensured that almost all cells would undergo phagocytosis resulted in the identification of a poorly responsive subpopulation of cells. The size of this poorly responsive subpopulation varied significantly between experiments, but the subpopulation generally appeared to involve the cells with a lower background level of H_2O_2. The identification of cellular heterogeneity on the basis of responsiveness to stimulation through complement receptors rather than more directly through stimulation with PMA fits well with the concept proposed above that some abscess cells may possess abundant CR3 molecules which are deactivated with respect to particular functions. Why this should be the case for only a proportion of the abscess cells is unclear.

It was possible to sort the abscess neutrophils which responded poorly to stimulation through CR3 and the abscess neutrophils which responded optimally and thus to observe which fraction contained the majority of abscess-derived bacteria. In all cases shown in Table 2, the number of abscess-derived bacteria in the low-fluorescence cell population was greater than the number of abscess-derived bacteria in the high-fluorescence group (2- to 11-fold). Therefore, it is possible that the poorly responsive group of abscess neutrophils contained a large proportion of the cells which had phagocytosed bacteria in vivo but which were subsequently unable to kill, either because of the poor respiratory burst or because of some inadequacy of CR3 receptors.

The generation of intracellular DCF depends to some extent on the activity of intracellular peroxidases, although the mechanism of this activity remains unclear. However, the subpopulation of abscess cells which we describe in this paper is unlikely to represent cells that contain significantly different absolute levels of myeloperoxidase, because stimulation with PMA induces an increase in intracellular DCF levels in all cells. However, it is possible that the cells that respond poorly to stimulation through CR3 are unable to release granule enzymes as effectively as the same cells stimulated with PMA. If this is the case, it could also explain the association between cells that respond poorly in the DCF assay and cells that contain the majority of abscess-derived bacteria. However, at present we do not have a fluorescence technique for quantitative measurement of myeloperoxidase activity in individual cells, which would permit the sorting of such cells.

Abscess neutrophils kill in vitro those bacteria which appear to be extracellular in an abscess (Finlay-Jones et al., in press). This indicates that the conditions within an abscess are not optimal for phagocytic killing of bacteria, and also that these conditions do not have an irreversible effect, at least on most of the abscess neutrophils. Indeed, almost all of the neutrophils are able to phagocytose efficiently in vitro (Table 1). However, this is in contrast to the intracellular persistence of bacteria in viable abscess-derived neutrophils. This persistence is evident under otherwise optimal conditions for phagocytic killing in vitro. The persisting intracellular bacteria are protected from antibiotic therapy and provide a continuing source of reinfection. Methods such as the method described in this report, which can enrich abscess neutrophils containing persisting bacteria, will be of value in solving these problems.

One of the difficulties associated with using the DCFH-DA method to measure H_2O_2 production in stimulated mouse neutrophils is the small increase in H_2O_2 content observed compared with the increase obtained with human peripheral blood neutrophils (2). Maximal stimulation of mouse abscess, bone marrow, or peritoneal exudate neutrophils with PMA is generally two- to threefold and never more than eightfold. Human neutrophils routinely produce a 20- to 40-fold increase in DCF content (and therefore H_2O_2 content) after PMA stimulation. Consequently, defining differentially activated subpopulations of mouse neutrophils, particularly for cell-sorting experiments, is more difficult. For this reason, an examination of alternative flow cytometric measurements of neutrophil function, such as membrane potential (8), pH (10), or Ca^{2+} level (11), may be of value.

The subpopulations of abscess neutrophils described in this report are not merely a reflection of the heterogeneous populations of neutrophils observed in peripheral blood of infected humans (1) by flow cytometry. In this phenomenon maximal stimulation of the respiratory burst with PMA has been shown to induce a bimodal pattern of H_2O_2 production in the peripheral blood neutrophil population. In our study of mouse abscess neutrophils it was only in the presence of bacteria and opsonins that the neutrophils responded differentially. Stimulation with PMA induced similar increases in intracellular H_2O_2 in all cells in the population.

We have not yet examined fully the use of abscesses induced with gram-positive organisms in the systems described above. However, it has been reported that neutrophils from chronic *S. aureus*-induced abscesses in rabbits show an overall reduction in respiratory burst activity compared with neutrophils from acute abscesses (D. Berger and B. Herndon, J. Leukocyte Biol. **47**:295, 1989). The phagocytic activities of the two populations were the same. Although individual neutrophils were not examined, the data support the concept of a population of neutrophils that are poorly responsive to opsonic activation of bactericidal functions and may contribute to the persistence of bacteria within chronic abscesses.

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