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# Determination of Active Phagocytosis of Unopsonized *Porphyromonas gingivalis* by Macrophages and Neutrophils Using the pH-Sensitive Fluorescent Dye pHrodo

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**Phagocytosis of pathogens is an important component of the innate immune system that is responsible for the removal and degradation of bacteria as well as their presentation via the major histocompatibility complexes to the adaptive immune system. The periodontal pathogen *Porphyromonas gingivalis* exhibits strain heterogeneity, which may affect a phagocyte's ability to recognize and phagocytose the bacterium. In addition, *P. gingivalis* is reported to avoid phagocytosis by antibody and complement degradation and by invading phagocytic cells. Previous studies examining phagocytosis have been confounded by both the techniques employed and the potential of the bacteria to invade the cells. In this study, we used a novel, pH-sensitive dye, pHrodo, to label live *P. gingivalis* strains and examine unopsonized phagocytosis by murine macrophages and neutrophils and human monocytic cells. All host cells examined were able to recognize and phagocytose unopsonized *P. gingivalis* strains. Macrophages had a preference to phagocytose *P. gingivalis* strain ATCC 33277 over other strains and clinical isolates in the study, whereas neutrophils favored *P. gingivalis* W50, ATCC 33277, and one clinical isolate over the other strains. This study revealed that all *P. gingivalis* strains were capable of being phagocytosed without prior opsonization with antibody or complement.**

In humans, chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth that results in destruction of the alveolar bone and other supporting tissue (1). It has been estimated to affect up to 25% of the dentate population, with severe forms affecting 5 to 6% (2, 3). While the etiology of chronic periodontitis is multifactorial, evidence suggests that the levels of specific Gram-negative bacteria in the subgingival plaque biofilm play a major role in the pathogenesis of the disease (4). Of these bacteria, *Porphyromonas gingivalis* is implicated as a major etiological agent (5). However, the presence of bacterial pathogens alone is not sufficient to cause the severe tissue and bone destruction seen in patients (6, 7). The host immune response to *P. gingivalis* and the release of its major virulence factors into gingival tissue are likely to be a major factor in inducing a chronic host inflammatory response, which in turn results in cumulative tissue destruction and alveolar bone resorption (4, 5, 8–11).

An important process of the innate immune response to bacteria is phagocytosis, a mechanism by which the host can eliminate invading microorganisms and alert other arms of the immune response. A large number of cell types are capable of phagocytosis to various degrees. Neutrophils and monocyte/macrophages are designated "professional phagocytes" due to their highly efficient ability to internalize a variety of targets (12). Phagocytosis is initiated by the interaction of cell surface receptors with ligands found on the bacteria, such as lipopolysaccharides, or host-derived opsonins, such as complement or IgG antibodies (13). One of the commonly observed characteristics in chronic periodontitis is the presence of macrophages in the localized diseased tissue (14, 15). For example, elevated numbers of macrophages have been detected in gingival tissue biopsy specimens from chronic periodontitis patients (14).

*P. gingivalis* appears to be able to invade human phagocytic cells through fimbria-mediated interactions with Toll-like receptor 2 (TLR2) and the β2 integrin CD11b/CD18 (16). This invasion may lead to intracellular survival of the bacteria either within the cytoplasm of the cell or within compromised endosomes that do not fuse with lysosomes (17).

Neutrophils play an important role in "homeostasis" of periodontal tissues with the bacterial biofilm. These cells make up the majority of the leukocytes recruited to the gingival crevice (18) and are believed to form a "defense wall" against the plaque biofilm (19). During periodontitis, neutrophils play an active role both in trying to rectify the dysbiosis and in promoting chronic inflammation (19). The antimicrobial mechanisms of neutrophils include phagocytosis and the release of reactive oxygen species, antimicrobial peptides, proteases, and neutrophil extracellular traps (NETs). In addition to these mechanisms, neutrophils are able to produce and secrete C-X-C and C-C chemokines as well as proinflammatory and anti-inflammatory cytokines (20, 21).

Measuring the ability of a macrophage or neutrophil to phagocytose *P. gingivalis* has been confounded by the labeling techniques employed and by the potential of the bacteria to invade host cells or evade phagocytosis. Most studies label *P. gingivalis* with fluorescein isothiocyanate (FITC) and then perform a quenching step to remove the fluorescence associated with surface-bound bacteria. This method is problematic in that there is no differentiation between bacteria that have been actively phago-

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cytosed and those that may have invaded the cytosol or prevented maturation and acidification of the phagosome. In this study, we use pHrodo-Red, a novel pH-sensitive fluorochrome that has been used to examine phagocytosis of bioparticles and apoptotic cells (22). Using pHrodo-Red-labeled *P. gingivalis*, we were able to determine if unopsonized bacteria are phagocytosed into an active phagosome, which has an acidic pH. Thus, fluorescence occurred only if the bacteria were in an acidic phagosome, while those found on the surface, on the cytosol, or in compromised endosomes could easily be excluded from analysis through a lack of fluorescence. Here, we determine the ability of mouse and human macrophages and mouse neutrophils to phagocytose a variety of unopsonized *P. gingivalis* strains and clinical isolates.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Porphyromonas gingivalis* strains W50 (ATCC 53978; fimbrial type IV), 33277 (ATCC 33277; fimbrial type I), and A7A1-28 (ATCC 53977; fimbrial type II) and clinical isolates 3A1 (fimbrial type II), 3-3, and 84-3 (the last two fimbrial type I), obtained from the Melbourne Dental School culture collection, were grown and harvested as previously described (9). The fimbrial genotypes were as designated by Amano et al. and Nagano et al. (23, 24). Bacteria were grown in batch culture in Todd-Hewitt broth (36.4 g/liter; Oxoid, Hampshire, England) supplemented with cysteine (1 g/liter; Sigma-Aldrich, NSW, Australia), hemin (5 mg/liter; Sigma-Aldrich), and menadione (1 mg/liter; Sigma-Aldrich). Cultures were grown in an MK3 Anaerobic Workstation (Don Whitley Scientific, NSW, Australia) at 37°C with a gas composition of 5% $H_2$ and 10% $CO_2$ in $N_2$ for 24 to 48 h. All bacteria were harvested during the late exponential phase as determined by the growth curve and optical density measured at 650 nm using a spectrophotometer (model Cary 50 Bio UV/Spectrophotometer; Varian, CA). Bacterial concentrations were determined using a live/dead fluorescence system. The green fluorescent DNA dye Syto9 (Life Sciences Pty Ltd., NSW, Australia) was used in conjunction with propidium iodide (PI; Life Sciences) to determine the quantity of viable bacteria, which were counted on the Cell Lab Quanta SC Flow Cytometer (Beckman Coulter Inc., NSW, Australia). The Quanta SC is equipped with an argon ion laser operating at an excitation wavelength of 488 nm with green fluorescence measured through a 525-nm filter (FL1) and red fluorescence measured through a 575-nm filter (FL2). When passaged on solid media, all species were grown on horse blood agar (HBA; 40 g/liter blood agar base no. 2, 10% [vol/vol] defibrinated horse blood, 1 mg/liter menadione) for 4 to 8 days.

**Labeling of bacteria.** *P. gingivalis* strains were labeled with pHrodo-Red succinimidyl ester (pHrodo; Life Sciences) as per the manufacturer's instruction with a slight modification. The manufacturer recommended washing the bacteria and poststaining with methanol, which we found caused severe clumping and death of the bacteria. As such, we used phosphate-buffered saline (PBS; Sigma) so as to not kill the bacteria and to avoid excessive aggregation. Bacteria were grown to late exponential phase, harvested by centrifugation (9,000 × *g*, 30 min, 4°C), and then washed (twice) with PBS. Bacteria were resuspended at $3 \times 10^9$ bacteria/ml in 100 mM sodium bicarbonate, pH 8.5, after harvesting and washing. pHrodo-Red was added to the bacteria at a concentration of 0.5 mM, and the mixture was incubated for 1 h at room temperature, with no light, and with gentle mixing. Labeled bacteria were then washed (3 times) with PBS to remove free dye and resuspended at $3 \times 10^9$ bacteria/ml in PBS. In order to confirm that all *P. gingivalis* strains were labeled with equivalent levels of pHrodo-Red, the mean fluorescence intensity was measured and found to be comparable at a range of pHs (data not shown). For the superresolution imaging, *P. gingivalis* W50 was labeled with Alexa-Fluor 488 (Life Sciences) as per the protocol described above. Viability postlabeling was determined by plating the bacteria at various dilutions using the drop plate method on horse blood agar (HBA), and CFU were enumerated after incubation (under anaerobic incubation at 37°C for 6 days). Sixty-five to 70% of the bacteria were determined to be viable postlabeling.

**Phagocytic cells.** RAW 264.7 (ATCC TIB-71) cells were used as murine macrophage cells (25) and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 25 mM L-glutamine, 10% (vol/vol) heat-inactivated fetal calf serum (FCS), and 100 IU/ml penicillin-streptomycin. THP-1 (ATCC TIB-202) cells were used as human monocytic cells (26) and cultured in RPMI 1640 medium containing 25 mM L-glutamine, 10% (vol/vol) heat-inactivated FCS, 100 IU/ml penicillin-streptomycin, and 0.05 mM 2-mercaptoethanol. Primary murine neutrophils were obtained from the peripheral blood of C57BL/6 mice. Briefly, blood was obtained from mice by cardiac puncture and combined with 50 μl of 6% EDTA (Sigma) per 1 ml of blood to prevent coagulation and kept at room temperature. Samples were diluted to 7 ml with PBS and laid over 3 ml of Histopaque 1077 (Sigma). The sample was centrifuged at 400 × *g* for 30 min at room temperature. The granulocyte and erythrocyte pellet was collected following centrifugation, peripheral blood mononuclear cells remaining at the Histopaque-PBS interface. Hypotonic shock with distilled water was used to rupture the erythrocytes for 30 s. The solution was then buffered with 5× PBS to prevent neutrophil lysis. The neutrophils were pelleted and resuspended in Hanks' balanced salt solution (HBSS; Sigma). The neutrophil purity of the final sample was determined to be >95% (data not shown) by staining with rat anti-mouse Ly6G FITC-conjugated antibody (BD Biosciences) and analysis by flow cytometry with Cytomics FC 500 (Beckman Coulter). All cells were counted using a Z1 Coulter Particle Counter (Beckman Coulter) and maintained at 37°C, 5% (vol/vol) $CO_2$. All animal usage was approved by the University of Melbourne Ethics Committee for Animal Experimentation.

**Phagocytosis assay.** Opsonization was examined using RAW 264.7 cells; pHrodo-Red-labeled *P. gingivalis* W50 was preincubated in antibiotic- and serum-free DMEM containing either 20% normal mouse serum (BALB/c) or 20 μg/ml of purified anti-RgpA-Kgp complex rabbit polyclonal antibody (pAb) IgG for 30 min at 37°C. The anti-RgpA-Kgp rabbit pAb IgG was generated by immunizing rabbits twice with RgpA-Kgp complex (50 μg, day 0 and day 30) in incomplete Freund's adjuvant (IFA; Sigma). The IgG was purified from sera (collected day 42) using a protein A/G column (Life Technologies). Excess opsonin was then removed by centrifugation (9,000 × *g*, 30 min, 4°C) before the bacteria were used in the assay. Adherent macrophages (RAW 264.7) were detached using 0.25% trypsin-EDTA and resuspended in antibiotic- and serum-free DMEM at $1.5 \times 10^6$ cells/ml. The nonadherent THP-1 cells and murine primary peripheral blood neutrophils were washed once and then suspended in antibiotic- and serum-free RPMI 1640 at $1.5 \times 10^6$ cells/ml. The assay was carried out in 96-well plates in a volume of 200 μl per well. pHrodo-Red-labeled bacteria were added in increasing ratios of bacteria to cell (bacterium-to-cell ratio [BCR]) and incubated for 1 h at 37°C, 5% (vol/vol) $CO_2$. After incubation, the cells were placed on ice to halt phagocytosis and then washed (twice in ice-cold PBS) and resuspended in ice-cold PBS for analysis by flow cytometry on the FC500 instrument (Beckman Coulter). A typical forward and side scatter gate was set to exclude dead cells and aggregates; a total of $3 \times 10^4$ events in the gate were collected, and phagocytosis was identified as pHrodo-Red-positive cells (pHrodo-Red fluorescence was measured using a 575-nm filter; FL2). As a negative phagocytic control, each bacterial strain and phagocytic cell combination was also incubated on ice. A one-way analysis of variance (ANOVA) (Dunnett's T3) test was used to determine statistical differences (GraphPad Prism Software version 5.04) and *P* values of $<0.05$ were considered significant.

**Superresolution imaging.** To visualize the phagocytosis of *P. gingivalis* W50 by RAW 264.7 macrophages, $1 \times 10^5$ RAW 264.7 cells/per slide were grown in chambered coverglass slides (Thermo Fisher Scientific, Scoresby, Victoria, Australia). The cells were washed with PBS (2 times) and incubated (1 h, 37°C) in the dark with Alexa-Fluor 488-labeled *P. gingivalis* W50 (ratio of bacteria to cells, 80:1) in serum- and antibiotic-free DMEM. Cells were then washed with PBS (once) and fixed with 4%

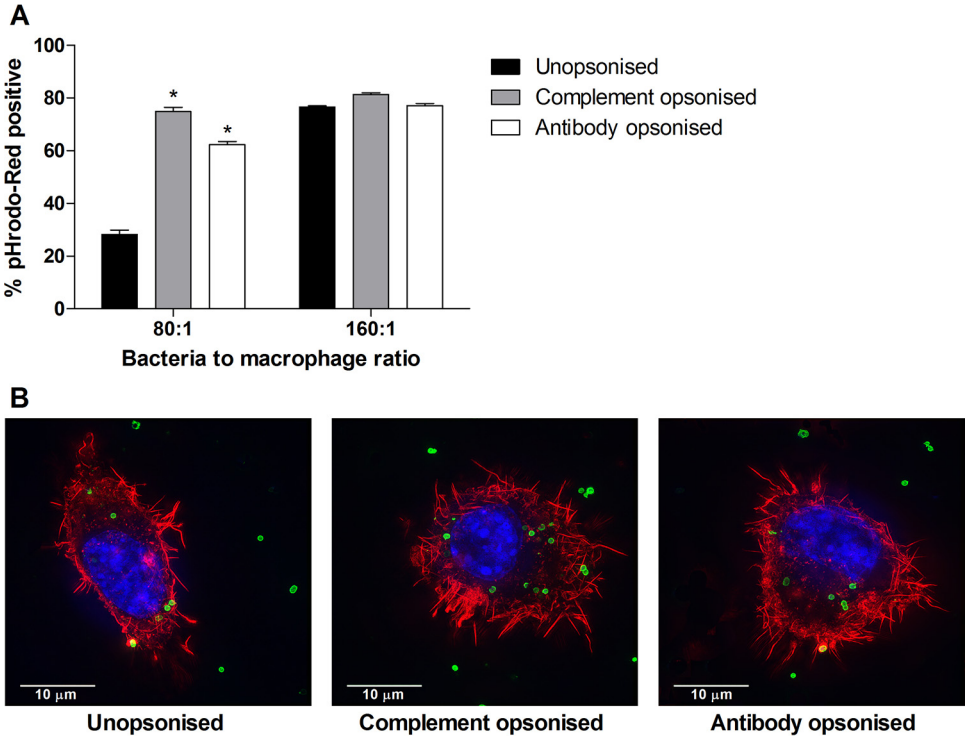


**FIG 1** Unopsonized *P. gingivalis* W50 is phagocytosed by macrophages. *P. gingivalis* W50 was labeled with pHrodo-Red and either left unopsonized or opsonized with 20% normal mouse serum (BALB/c; complement opsonization) or 20 μg/ml anti-RgpA-Kgp complex pAb (antibody opsonization) in antibiotic- and serum-free DMEM. Bacteria were added at a ratio of 80:1 or 160:1 to macrophages (RAW 264.7). (A) The macrophages were able to phagocytose both opsonized and unopsonized *P. gingivalis*. Data are expressed as the percentages of cells that are pHrodo-Red positive. Values are means ± standard errors of the means (SEM); *, $P < 0.05$ versus unopsonized. (B) To visualize this phagocytosis, Alexa Fluor 488-labeled *P. gingivalis* was used instead of pHrodo-Red at a BCR of 80:1, and fluorescence microscopy was performed. The sliced images were obtained from a section with a defined size (10 to 50 z stacks). *P. gingivalis* W50 (green) can be seen within the actin-stained (red) macrophages with nuclei stained with DAPI (blue).

paraformaldehyde for 5 min at room temperature (RT). The cells were washed with PBS (2 times) and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Pty. Ltd., New South Wales, Australia) in PBS at RT. Following washing with PBS (2 times), the cells were blocked with 1% bovine serum albumin (BSA) in PBS at RT for 5 min. The actin filaments were stained with phalloidin-tetramethyl rhodamine isocyanate (phalloiding-TRITC; Sigma-Aldrich Pty. Ltd., New South Wales, Australia) at 5 μg/ml in 1% BSA–PBS for 40 min at 37°C, and nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole) as per the manufacturer's instruction (Life Technologies). The cells were stored in SlowFade Diamond Antifade Mountant (Life Technologies) before imaging on the Deltavision OMX Structured Illumination Microscope V4 Blaze (Applied Precision, WA, USA). Images were produced using the Fiji imaging processing package (27).

## RESULTS

**Unopsonized *P. gingivalis* W50 is phagocytosed by mouse macrophages.** Opsonization is an important process that enhances the innate immune response against bacteria. *P. gingivalis* has been shown to degrade common opsonins such as antibody and complement (28, 29). To investigate whether opsonization is important for phagocytosis of *P. gingivalis*, we initially labeled *P. gingivalis* strain W50 with the pH-sensitive dye pHrodo-Red. Labeled *P. gingivalis* cells were then preincubated in serum-free, unopsonized medium, medium containing 20% normal mouse serum (complement opsonization), or medium containing 20 μg/ml of anti-RgpA-Kgp complex pAb (antibody opsonization) and then with the macrophages in antibiotic- and serum-free medium for 1 h before being placed on ice to halt phagocytosis. The macrophages were then examined by flow cytometry for pHrodo-Red fluorescence, a measure of *P. gingivalis* in an acidified phagosome. Opsonization with either normal mouse serum (complement) or a pAb against *P. gingivalis* was found to enhance phagocytosis in murine macrophages (RAW 264.7) compared with unopsonized bacteria at a BCR of 80:1 (Fig. 1A). However, at a higher BCR (160:1) unopsonized bacteria were phagocytosed at a level similar to that of opsonized bacteria (Fig. 1A). Using high-resolution microscopy and Alexa Fluor 488-labeled *P. gingivalis* W50 (green) at a BCR of 80:1, it was observed that unopsonized *P. gingivalis* W50 cells were phagocytosed by a macrophage (stained for actin in red and for DAPI in blue) at a level similar to that of serum- and pAb-opsonized bacteria (Fig. 1B).

**Macrophage phagocytosis of pHrodo-Red-labeled *P. gingivalis*.** The above data show that unopsonized *P. gingivalis* W50 is able to be phagocytosed. To examine whether this is restricted to one strain and the host cell, we then investigated the ability of mouse and human macrophages to phagocytose a range of unopsonized *P. gingivalis* strains and clinical isolates using the above method and pHrodo-Red-labeled bacteria. All strains of *P. gingivalis* were phagocytosed by both the mouse macrophage cell line RAW 264.7 and the human monocytic cell line THP-1. Phagocytosis was measured as an increase in pHrodo-Red fluorescence above the relevant ice control; phagocytosis is prevented by incubating the sample on ice for 1 h instead of at 37°C (Fig. 2A and 3A).

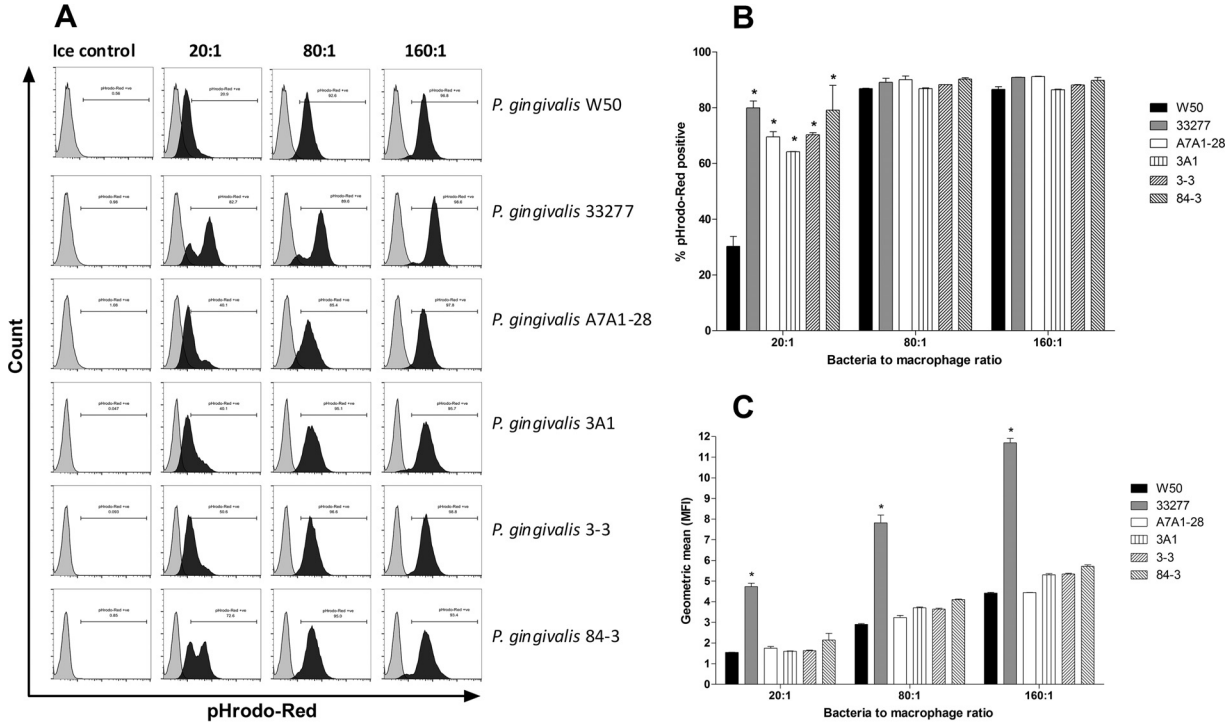


**FIG 2** Phagocytosis of *P. gingivalis* strains by the murine macrophage cell line RAW 264.7. *P. gingivalis* strains W50 (ATCC 53978), 33277 (ATCC 33277), and A7A1-28 (ATCC 53977) and clinical isolates 3A1, 3-3, and 84-3 were labeled with pHrodo-Red and examined for their ability to be phagocytosed by mouse macrophage cells (RAW 264.7) at increasing bacterium-to-cell ratios. Mouse macrophages are able to phagocytose all strains examined as determined by an increase in pHrodo-Red fluorescence (A). (B and C) Phagocytosis was quantitated by both the percentage of macrophages that are pHrodo-Red positive (B) and the MFI (C). Data are expressed as means ± SEM; *, $P < 0.05$ versus 33277, A7A1-28, 3A1, 3-3, and 84-3 (B), *, $P < 0.05$ versus W50, A7A1-28, 3A1, 3-3, and 84-3 (C). $n = 3$.

By examining the percentage of cells that were pHrodo-Red positive and the mean fluorescence intensity (MFI), it was possible to determine differences in "phagocytic ability" between phagocytic cell types and the different *P. gingivalis* strains. Mouse macrophages were highly phagocytic, with >80% of all cells pHrodo-Red positive at a BCR of >80:1. At a BCR of 20:1, *P. gingivalis* W50 was found to be phagocytosed significantly less than the other strains examined (Fig. 2B). Macrophages incubated with *P. gingivalis* ATCC 33277 had a significantly higher MFI than that of macrophages incubated with other *P. gingivalis* strains, suggesting that more bacteria per cell were phagocytosed (Fig. 2C). The human monocyte cell line THP-1 appeared to be less efficient at phagocytosing *P. gingivalis* at the low BMR of 20:1 and showed greater variability in pHrodo-Red-positive cells between the different *P. gingivalis* strains (Fig. 3B). THP-1 cells were also found to phagocytose more *P. gingivalis* ATCC 33277 organisms, as indicated by the significantly higher MFI at all BCRs examined (Fig. 3C).

**Neutrophil phagocytosis of pHrodo-Red-labeled *P. gingivalis*.** Neutrophils are a major phagocytic cell of the innate immune system, and several studies have shown that they are the predominant gingival cell infiltrate in periodontitis patients. Using the method developed for macrophages, we then examined the ability of murine neutrophils to phagocytose unopsonized *P. gingivalis* strains. Murine peripheral blood neutrophils were able to phagocytose all unopsonized *P. gingivalis* strains and clinical isolates examined, as measured by an increase in pHrodo-Red fluorescence above the ice control background (Fig. 4A). At a BCR of 80:1, 100% of the neutrophils were pHrodo-Red positive for all *P. gingivalis* strains examined (Fig. 4B). Although neutrophils were able to phagocytose all *P. gingivalis* strains and clinical isolates, there was a preference for *P. gingivalis* strains W50 and ATCC 33277 and clinical isolate 84-3, as indicated by the higher MFI (Fig. 4C). At a BCR of 20:1, *P. gingivalis* W50 and ATCC 33277 were phagocytosed more than all other strains in terms of the percentage of positive neutrophils (Fig. 4B). The MFI at 20:1 showed that ATCC 33277 was phagocytosed to a greater extent than the other bacterial strains. At 80:1, all neutrophils were positive for phagocytosis and all *P. gingivalis* strains tested were being actively phagocytosed, with the MFI showing strains W50, ATCC 33277, and 84-3 being phagocytosed to the greatest extent (Fig. 4C). Increasing the BCR to 160:1 resulted in an increase in the number of all bacteria being phagocytosed compared with 80:1; however, *P. gingivalis* strains W50, ATCC 33277, and 84-3 were phagocytosed at higher levels.

## DISCUSSION

Phagocytosis is a critical element of the innate immune response whereby invading pathogens are recognized, engulfed, degraded, and subsequently presented to the adaptive immune system via the major histocompatibility complexes. When pathogens are ingested, the resulting phagosome undergoes a series of fission and fusion events that modify the composition of their membrane and their contents, bestowing the phagosome with degradative properties. One of the major markers of phagosome maturation is the acidification of its interior, a process that aids in degradation (30).

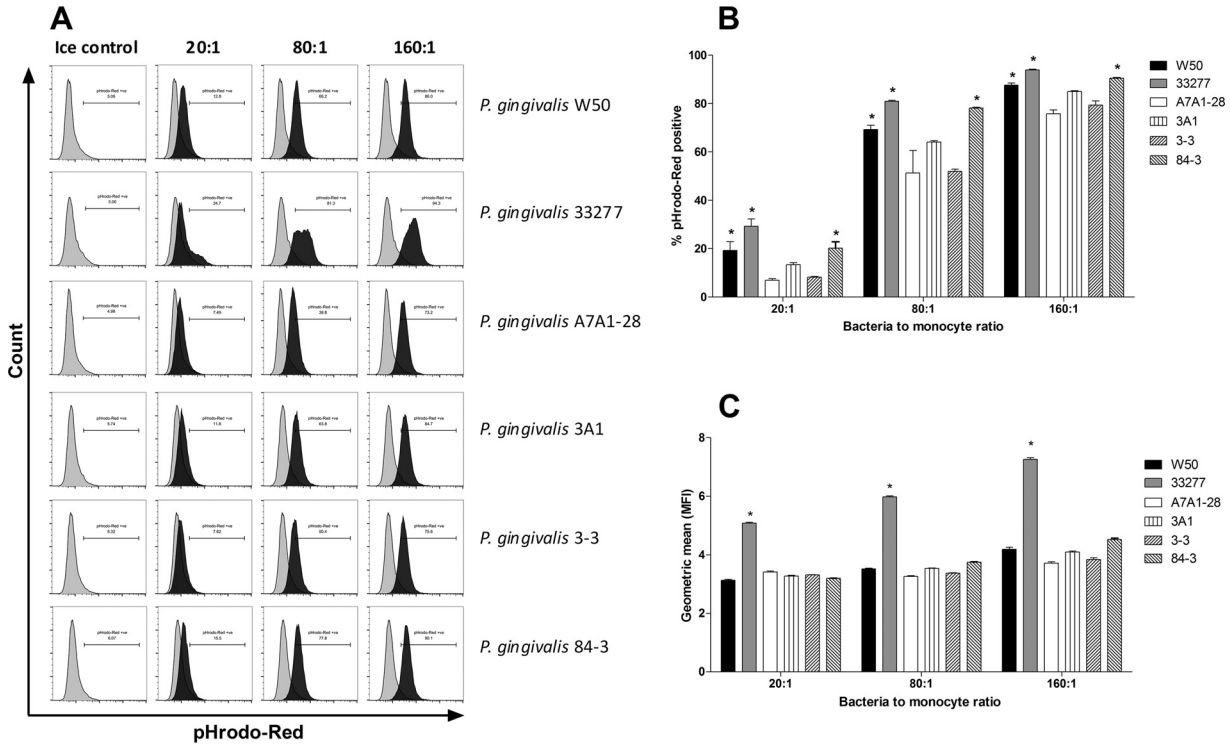


**FIG 3** Phagocytosis of *P. gingivalis* strains by the human monocytic cell line THP-1. *P. gingivalis* strains W50 (ATCC 53978), 33277 (ATCC 33277), and A7A1-28 (ATCC 53977) and clinical isolates 3A1, 3-3, and 84-3 were labeled with pHrodo-Red and examined for their ability to be phagocytosed by human monocytic cells (THP-1) at increasing bacterium-to-cell ratios. (A) Human monocytes are able to phagocytose all strains examined as determined by an increase in pHrodo-Red fluorescence. (B and C) Phagocytosis was quantitated by both the percentage of macrophages that were pHrodo-Red positive (B) and the MFI (C). Data are expressed as means ± SEM; *, $P < 0.05$ versus A7A1-28 and 3-3 (B); *, $P < 0.05$ versus W50, A7A1-28, 3A1, 3-3, and 84-3 (C). $n = 3$.

Some bacteria, however, have developed the ability to either survive within the acidified environment, such as *Salmonella enterica* serovar Typhimurium (31), or prevent acidification (32). Numerous studies have examined the ability of the two major phagocytic cells associated with periodontitis, neutrophils and macrophages, to phagocytose periodontal pathogens, in particular, *P. gingivalis*. These studies have employed various methods to investigate phagocytosis; however, each method has limitations. A common method consists of labeling bacteria with fluorescent dyes such as fluorescein isothiocyanate (FITC) and examining phagocytic cells for fluorescence by flow cytometry (33, 34). While this method is relatively fast, it is not without limitations, because cell surface-bound bacteria must be quenched before quantitation; additionally, bacterial invasion and phagocytosis are indistinguishable. Furthermore, FITC fluorescence decreases under acidic conditions, further compromising this method. Microscopy of fixed and stained cells has been used to distinguish invasion from phagocytosis either by fluorescence microscopy (35) or by transmission electron microscopy (17). Both methods take time and can be costly, prohibiting fast throughput of results, and may not show phagocytosis of bacteria in a maturing phagosome.

We show that using the pH-sensitive pHrodo dye it is possible to measure the active phagocytosis of *P. gingivalis*. Initially inactivated, pHrodo-labeled *Escherichia coli* was used as phagocytic "bioparticles" to measure a cell's ability to phagocytose. Recently, pHrodo has been used to label other particles of interest, such as heat-killed bacteria (36), lipids and proteins (37), UV polystyrene beads to track phagolysosome acidification (38), dead neural stem cells (39), and nanoparticles (40). In this study, we show that live *P. gingivalis* strains can be labeled with pHrodo-Red. The ability to study phagocytosis of live bacteria is of particular significance, as heat-killed, nonviable bacteria are phagocytosed at a lower rate than live bacteria (41). Thus, such assays may not reflect the true nature of phagocytic cell-bacterium interactions *in vivo*.

Opsonization of bacteria is often required to induce or enhance phagocytosis. The complement system is particularly important in this process, with deposition of C3b on the bacterial surface interacting with CR1 and Fc receptors on phagocytic cells. IgG binding to bacterial surfaces also enhances phagocytosis by interacting with Fc receptors. *P. gingivalis* has been shown to degrade IgG and complement C3 and C5, thus preventing deposition of C3b (28, 29). This degradation has been hypothesized as a mechanism whereby *P. gingivalis* evades the immune response, in particular, phagocytosis. The results presented in the current study show that phagocytic cells do not require opsonization of *P. gingivalis* in order to be recognized and phagocytosed (Fig. 1). Heat-killed or nonviable *P. gingivalis* may not retain the ability to degrade opsonins; thus, the use of live bacteria in this study enables a closer comparison to phagocytosis during infection *in vivo*. It should also be noted that this study was conducted in the absence of FCS, in order to remove the possibility of exogenous proteins coating the bacteria and increasing/decreasing phagocytosis, as has been previously reported (42)

This study revealed that all *P. gingivalis* strains examined were

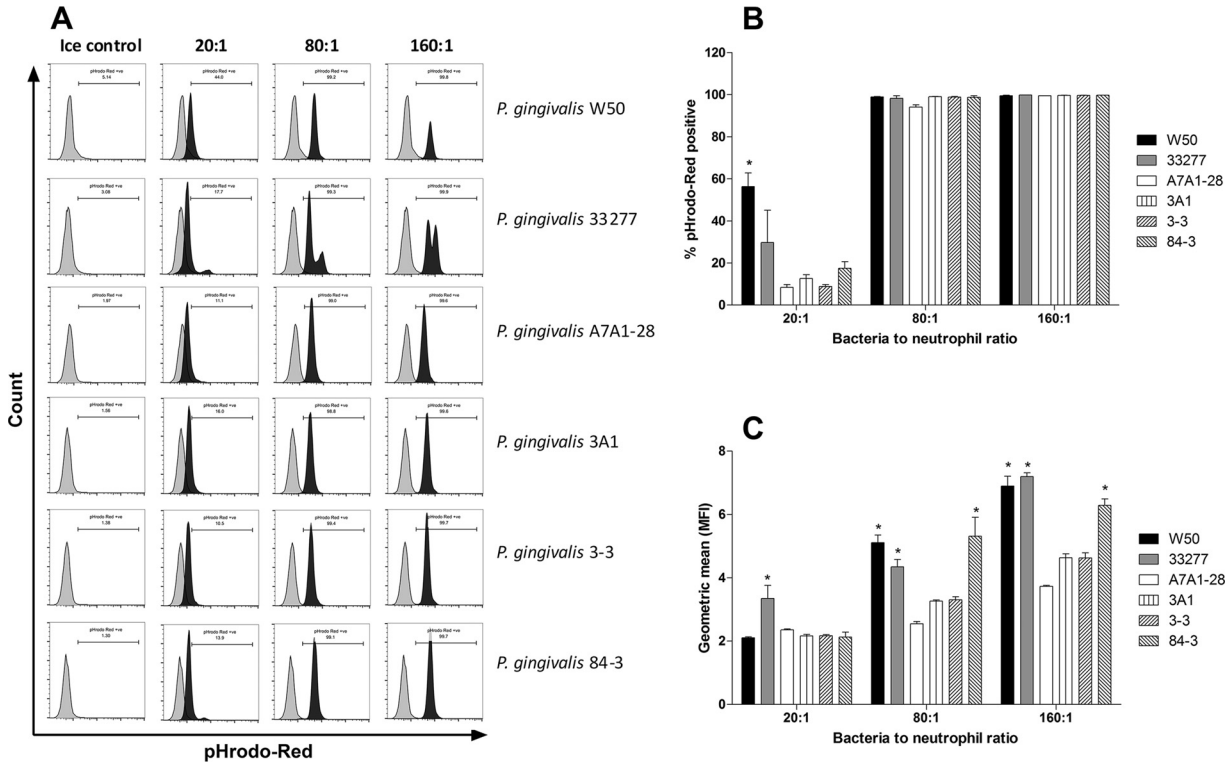


**FIG 4** Phagocytosis of *P. gingivalis* strains by murine peripheral blood neutrophils. *P. gingivalis* strains W50 (ATCC 53978), 33277 (ATCC 33277), and A7A1-28 (ATCC 53977) and clinical isolates 3A1, 3-3, and 84-3 were labeled with pHrodo-Red and examined for their ability to be phagocytosed by murine peripheral blood neutrophils at increasing bacterium-to-cell ratios. (A) Mouse neutrophils are able to phagocytose all strains examined as determined by an increase in pHrodo-Red fluorescence. (B and C) Phagocytosis was quantitated by both the percentage of macrophages that were pHrodo-Red positive (B) and the MFI (C). Data are expressed as means ± SEM; *, $P < 0.05$ versus A7A1-28, 3A1, 3-3, and 84-3 (B); *, $P < 0.05$ versus A7A1-28, 3A1, and 3-3 (C). $n = 3$.

actively phagocytosed by macrophages and neutrophils without the need for opsonization. By examining the percentage and MFI of pHrodo-Red, we were able to detect differences in the ability of macrophages and neutrophils to phagocytose different *P. gingivalis* strains. These differences may be attributed to differences in the capsule, outer membrane, or fimbriae. Macrophages exhibited a preference for the atypically fimbriated strain *P. gingivalis* ATCC 33277. The strain 33277 is atypical in that its fimbriae are very long, and this elongation has been attributed to a mutation in the *fimB* gene, which controls the length of the fimbriae (24). This mutation is unique to this strain and was not present in the other strains tested (our unpublished data). *P. gingivalis* ATCC 33277 exhibited a significantly higher MFI than all other strains tested in macrophages (Fig. 2C and 3C). The elongated fimbriae may be actively binding to receptors on the macrophage surface, facilitating greater internalization (16). Fimbrial protein binding to complement receptor 3 has been identified as a mechanism by which *P. gingivalis* can invade macrophages and evade killing (43). Complement receptor 3-mediated internalization of pathogens is known to lead to a less robust microbicidal mechanism in both macrophages and neutrophils (44, 45). This suggests that invading *P. gingivalis* may evade low pH phagolysosomes via this mechanism. Interestingly, the MFI pattern was slightly different in neutrophils, although *P. gingivalis* strain ATCC 33277 was still among the more highly phagocytosed strains (Fig. 4C).

In this study, we have demonstrated for the first time a novel method for examining phagocytosis of the periodontal pathogen *P. gingivalis*. Utilizing the pH-sensitive pHrodo dye, it was possible to label live *P. gingivalis* strains and observe the ability of various phagocytic cells to phagocytose the bacteria. This method is fast and reliable, does not require additional quenching steps, and measures only bacteria taken into an acidifying phagosome, thus yielding a true measure of phagocytosis.

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