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


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.
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 AT81-28 (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).

Bacterial strains 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% H2 and 10% CO2 in N2 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 collected, and phagocytosis was identified as pHrodo-Red-positive cells.

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 × 108 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 × 109 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 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 Histopoque-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 Hank’s 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) CO2. 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 × 106 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 × 106 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) CO2. 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 × 104 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 × 106 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%
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 (phalloidin-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 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).

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).
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). 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). 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).
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-bacteria 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...
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.

ACKNOWLEDGMENTS

Superresolution imaging was performed by Ben Hibbs at the Materials Characterization and Fabrication Platform (MCFP) at the University of Melbourne. Katrina Laughton is thanked for technical assistance.

FUNDING INFORMATION

This work was funded by the Australian Government Department of Industry, Innovation and Science (Cooperative Research Centre grant 20080108) and a National Health and Medical Research Council project grant (APP1029878).

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