Infection and Immunity

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Oxidant Generation by Single Infected Monocytes after Short-Term Fluorescence Labeling of a Protozoan Parasite[▽][†]

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Leishmania spp. are intracellular protozoa residing in mononuclear phagocytes. *Leishmania* organisms are susceptible to microbicidal responses generated in response to phagocytosis. Assuming that both phagocyte and parasite populations are heterogeneous, it is advantageous to examine the response of individual cells phagocytosing living parasites. Because *Leishmania* spp. lose virulence during the raising of transfectants, we developed a method to label live *Leishmania chagasi* short-term with fluorescent dyes. Up to six parasite divisions were detected by flow cytometry after labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE), dioctadecyl-tetramethylindo carbocyanine perchlorate, or chloromethyl tetramethylrhodamine. Labeled parasites entered mononuclear phagocytes as determined by confocal and time-lapse microscopy. Dihydroethidium (DHE) was used to detect macrophage-derived oxidants generated during phagocytosis. Presumably *Leishmania* organisms are opsonized with host serum/tissue components such as complement prior to phagocytosis. Therefore, we investigated the effects of opsonization and found that this increased the efficiency of CFSE-labeled parasite entry into monocytes (84.6% ± 8.8% versus 20.2% ± 3.8% monocytes infected; *P* < 0.001). Opsonization also increased the percentage of phagocytes undergoing a respiratory burst (66.0% ± 6.3% versus 41.0% ± 8.3% of monocytes containing CFSE-labeled parasites; *P* < 0.001) and the magnitude of oxidant generation by each infected monocyte. Inhibitor data indicated that DHE was oxidized by products of the NADPH oxidase. These data suggest that opsonized serum components such as complement lead to more efficient entry of *Leishmania* into their target cells but at the same time activate the phagocyte oxidase to generate microbicidal products in infected cells. The parasite must balance these positive and negative survival effects in order to initiate a viable infection.

Leishmania protozoa are intracellular parasites residing in macrophages or other mononuclear cells of their mammalian hosts (13). The parasites are inoculated into mammalian cutaneous tissue in the form of a promastigote, which must locate an appropriate phagocyte and stimulate its own internalization in order to survive. However, promastigotes are susceptible to killing by the reactive oxygen species generated by the macrophage during phagocytosis (19, 20, 27). Once intracellular, parasites convert to the more resistant obligate intracellular amastigote form. The *Leishmania* spp. have developed a number of mechanisms to protect themselves from oxidant-mediated killing while in their susceptible promastigote form. These include a surface glycolipid coat called lipophosphoglycan, antioxidant defense molecules such as iron superoxide dismutase, peroxidoxins, and a unique thiol-reducing factor, trypanothione (16, 33, 34). Furthermore, *Leishmania* spp. have the ability to suppress gamma interferon (IFN-γ)-mediated activation of macrophages through enhancing macrophage phosphatase activity (9, 30).

The redundant antioxidant and macrophage-deactivating mechanisms of the parasite must compete against potent microbicidal activities of the phagocyte. Studies of cultured human and murine monocytes and macrophages demonstrate that a respiratory burst occurs after phagocytosis of *Leishmania* promastigotes, resulting in superoxide and hydrogen peroxide production (5, 10, 31). These reports, however, do not indicate the proportion of infected macrophages that produce oxidants and whether some parasites reside in nonactivated cells. These questions require a method to detect oxidant production from single infected cells. However, the generation of fluorescently labeled *Leishmania* is complicated by the fact that transfection and expansion of mutants expressing exogenous markers takes weeks to months to accomplish (12, 15). Over this period of time many *Leishmania* spp., such as *L. chagasi* and *L. donovani*, down-modulate expression of many virulence characteristics (4). In order to detect oxidants produced by single cells during phagocytosis of virulent *Leishmania* promastigotes, we developed a method for short-term fluorescence labeling of *Leishmania* parasites and detection either by flow cytometry or confocal microscopy. The method allows the study of all parasite strains and lines without the loss of virulence or antigen expression. We discovered that the majority of human monocytes ingesting serum-opsonized *L. chagasi* undergo a respiratory burst, detected by the fluorescent dye dihydroethidium (DHE). In contrast, only a subset of monocytes produced oxidants upon phagocytosis of control

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nonopsonized parasites. *Leishmania* spp. are presumably rapidly opsonized with serum components including complement soon after inoculation into a host, and C3 opsonization has been found to enhance survival of *L. major* (24). These data suggest that opsonized serum components enhance parasite entry into monocytes but also enhance microbicidal activity of monocytes against invading *L. chagasi*.

MATERIALS AND METHODS

Human peripheral blood monocytes and macrophage lines. Mononuclear cells were isolated from the peripheral blood of normal healthy human donors by density sedimentation in Ficoll-Hypaque 1077 (Sigma Chemical Co., St. Louis, MO) (45). Monocytes were separated by adherence to six-well plates (flow cytometry) for 2 to 3 h at 37°C and 5% CO_2 in RPMI 1640 with 10% fetal calf serum (Gibco, Invitrogen Corporation, Carlsbad, CA) plus 50 μg of streptomycin/ml and 100 U of penicillin/ml (RP10). Monocytes were further purified by negative selection using the Human Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA) for flow cytometric or microscopic assays. The kit removes non-monocytes recognized by antibodies to CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A by using a magnetic column. The resultant monocyte preparation consists of 85.4% ± 4.0% CD14-positive cells according to flow cytometry ($n = 3$). For time-lapse video microscopy, RAW 264.7 cells were plated on poly-L-lysine-coated coverslips and exposed to *L. chagasi* promastigotes at a 5:1 ratio.

***Leishmania* cultivation and staining.** A Brazilian strain of *Leishmania chagasi* (MHOM/BR/00/1669) was maintained by serial passage in male Syrian hamsters as described previously (45). Promastigotes were cultured at 26°C in hemoflagellate modified minimal essential medium and used in the stationary phase of growth for all experiments (2).

Parasites in late logarithmic or stationary phase were labeled with one of three fluorescent stains. Promastigotes were incubated in 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min, in 5 μM chloromethyl tetramethylrhodamine (CMTMR) (Molecular Probes) for 20 min, or in 5 μM dioctadecyl-tetramethylindo carbocyanine perchlorate (CM-DiI) for 30 min in a 37°C water bath. After two washes, parasites were resuspended either at 2×10^7/ml in Hanks balanced salt solution or at 1×10^6/ml in hemoflagellate modified minimal essential medium for cultivation. Promastigotes were either fixed in 2% paraformaldehyde for flow cytometric analysis or opsonized with in 5% A/J serum at 37°C for 10 min and washed twice before use in infection studies.

Monocyte infections and detection of monocyte-derived oxidants. Adherent monocytes were infected with fluorescent promastigotes at 5:1 unless stated otherwise. Infections were synchronized by centrifugation (700 rpm, 5 min, 4°C) and transfer to 37°C and 5% CO_2. After 5 to 25 min, 5 μM DHE (Molecular Probes) was added and left for an additional 10 min in 37°C culture, after which promastigotes were removed by rinsing and phagocytes were detached in citric saline (0.135 M KCl, 0.015 M Na citrate). Cells were fixed in 2% paraformaldehyde in phosphate-buffered saline prior to flow cytometric analysis. Some monocytes were preincubated for 16 h with various concentrations of transforming growth factor β1 (TGF-β1) (R&D Systems, Minneapolis, MN) before infection with promastigotes and DHE staining. Other macrophages were exposed to IFN-γ (400 units; eBioscience) and/or lipopolysaccharide (LPS) (1 ng/ml; Sigma) 24 h prior to DHE staining.

Microscopic assay of parasite load. Monocytes or macrophages were infected, incubated with DHE, and detached in citric saline as described above. Cells were suspended at 1×10^6 cells/ml in Hanks balanced salt solution and applied to a slide by using a cytocentrifuge (500 rpm, 5 min). Slides were fixed and stained with Diff Quik stain, and cell-associated parasites were enumerated.

Flow cytometry. Cells were fixed in 2% paraformaldehyde in phosphate-buffered saline and then analyzed by flow cytometry (BD Biosciences, San Diego, CA). Ten thousand events were examined. The appropriate gate for monocytes or macrophages was determined by forward and side scatter and validated by anti-CD14 staining (phycoerythrin-Cy7; BD Biosciences PharMingen). Fluorescence was monitored at 480/30 nm (green CFSE) or 580/42 nm (red CM-DiI, CMTMR, and DHE). Data were analyzed using Cell Quest or Flow Jo software (BD Biosciences). Analyses were carried out on Becton Dickinson FACSCalibur equipped with an argon laser (488 nm). Data analysis was performed on fluorescence intensities. Cell Quest analysis software was used for fluorescence determination and data analysis.

Confocal microscopy. Fluorescently labeled promastigotes were incubated at a 5:1 ratio with differentiated U937 cells or human monocytes on glass coverslips
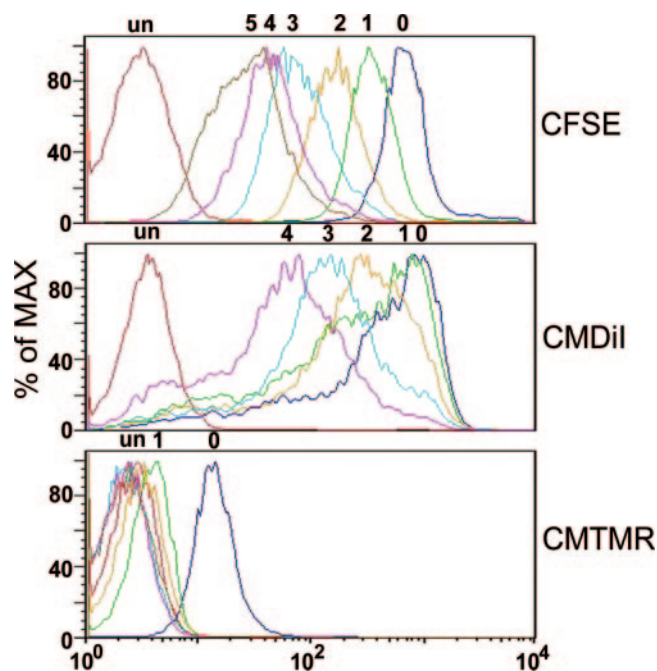

FIG. 1. Flow cytometry plot of fluorescently stained promastigotes during serial cell divisions. Promastigotes were stained with CFSE, CM-DiI, or CMTMR and cultured at 26°C. On days 0 through 5 of culture, aliquots were processed for flow cytometric analysis. Overlapping flow cytometry plots show the distribution of stain to daughter cells during five sequential days of growth. 0, promastigote sample prepared immediately after staining; un, unstained promastigotes. Peaks shifted to the left of the day 0 sample signify parasite scans on the indicated days.

for 30 min. Coverslips were mounted in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Dual-channel images were taken with a confocal laser scanning microscope (LSM510; Zeiss).

Time-lapse video microscopy. The RAW264.7 murine macrophage cell line was cultured on glass coverslips in six-well plates at 37°C in an atmosphere of 5% CO_2. Macrophages were stained with CMTMR (25 μM), and *L. chagasi* promastigotes were stained with CFSE (5 μM). Parasites and RAW264.7 cells were cocultured at 37°C for 100 min. Images were collected every 10 seconds for 16 min with a Ziess 510 inverted confocal microscope using a 63× objective lens.

Statistical analysis. Statistical analysis of data was done by one-way analysis of variance (ANOVA) followed by the Bonferroni test, using Sigma Stat software (Systat Software, Inc.).

RESULTS

Short-term fluorescent labeling of *Leishmania* promastigotes and detection by flow cytometry or confocal microscopy. We determined whether *L. chagasi* promastigotes could be detected after short-term labeling with fluorescent markers. Figure 1 shows flow cytometry plots of virulent promastigote cells stained in late logarithmic phase with one of three fluorescent dyes. Cells were fixed either immediately after staining (day 0) or after 1 to 5 days of culture at 26°C. Several points are worthy of note. First, unstained promastigotes did not exhibit sufficient autofluorescence to interfere with labels. Second, parasite divisions could be detected approximately every 24 h, a rate that conforms with the growth rate observed by other methods. Third, fluorescent labels are maintained even after fixation for flow cytometry, in contrast to the case for green fluorescent protein (GFP) label, which diminishes after fixa-
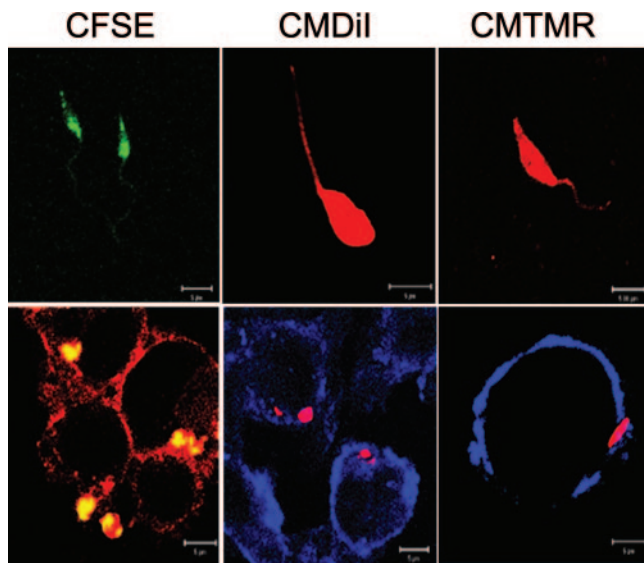
FIG. 2. Confocal laser scanning microscopic images of transiently stained promastigotes (top row) or of the macrophage cell line U937 (CFSE stain) or human monocytes (CM-DiI and CMTMR stains) infected with fluorescently labeled promastigotes (bottom row). Promastigotes were stained with 5 μM CFSE (green), 5 μM CM-DiI (red), or 5 μM CMTMR (red) as indicated. Differentiated adherent U937 cells were allowed to phagocytose promastigotes for 30 min and then stained with fluorescent phalloidin to mark the cytoskeletal actin. Actin stains were either tetramethyl rhodamine isocyanate-phalloidin (red) as a counterstain for green CFSE-labeled promastigotes or fluorescein isothiocyanate-phalloidin (green) as a counterstain for red CM-DiI- or CMTMR-labeled promastigotes.
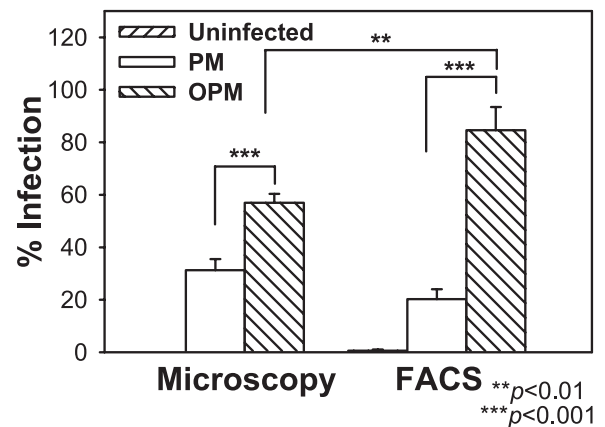

FIG. 3. Comparison between flow cytometric and microscopic assays for determining the percentage of cells infected with fluorescent *L. chagasi*. The percentage of column-purified monocytes infected with CFSE-labeled *L. chagasi* promastigotes 20 min after infection was determined both by flow cytometry and by microscopic assay. Promastigotes were either opsonized with C5-deficient serum (OPM) or remained nonopsonized (PM). Cytospin preparations were performed on the same cells as used in the flow cytometric assay and were stained with Wright Giemsa stain. Data represent the means and standard deviations from six separate experiments, with duplicate coverslips prepared for microscopy. Statistical analyses were done using the *t* test.

tion (40). Fourth, the most uniform staining was achieved with CFSE labeling, and parasites retained detectable label out to 5 days(divisions)afterlabeling.Stationary-phasevirulentpromastigotes, attenuated *L. chagasi* promastigotes, *L. donovani* promastigotes, gene knockout *L. donovani*, and axenic *L. chagasi* amastigotes could also be labeled efficiently with CFSE (data not shown). CFSE stain did not interfere with parasite motility or replication (data not shown). These results indicate that *Leishmania* spp. can be short-term labeled with either CFSE or CM-DiI and detected by flow cytometry.

Promastigotes stained with each of the above-mentioned dyes retained their label after infection of macrophages and could be visualized by confocal microscopy (Fig. 2). The human U937 cell line was incubated with labeled promastigotes under conditions favoring parasite phagocytosis, after which macrophage actin was stained with tetramethyl rhodamine isocyanate- or fluorescein isothiocyanate-labeled phalloidin to outline the cytoskeleton. Intracellular CFSE-labeled *Leishmania* remained fluorescent and detectable more than 48 h after phagocytosis. CFSE-labeled promastigotes could also be visualized after phagocytosis by primary murine bone marrow-derived macrophages, mouse peritoneal macrophages, murine or human cell lines, or human monocytes (data not shown).

Microscopy versus fluorescence for parasite quantification. We compared standard light microscopy with flow cytometry for quantification of human monocyte infections with promastigotes that were opsonized with serum or nonopsonized (Fig. 3). At 20 minutes after infection, more opsonized promasti-

gotes than nonopsonized promastigotes were internalized by monocytes ($P < 0.001$). Parasite loads were slightly higher when quantified by flow cytometry than when quantified by microscopy ($P < 0.01$). To investigate this discrepancy, we visualized the initial moments of parasite-monocyte interaction by using time-lapse confocal microscopy of fluorescent promastigotes and macrophages (see the supplemental material). Promastigotes (nonopsonized) were stained with CFSE, and the RAW 264.7 macrophage cell line was stained with the cytoplasmic dye CMTMR (Molecular Probes). Within minutes after phagocytosis, some CFSE-labeled promastigotes lysed and released dye into the macrophage, and the macrophage remained CFSE positive for a short period after parasite lysis. We hypothesized that these lysed intracellular parasites may lose morphological characteristics more quickly than monocytes lose the CFSE stain (see, as an example, the lower parasite in the supplemental material), accounting for the discrepancy between parasite loads in Fig. 3. To determine whether some infected monocytes detected by flow cytometry no longer contained morphologically recognizable parasites, we employed fluorescence-activated cell sorting to recover CFSE-positive monocytes at 25 min after phagocytosis of opsonized CFSE-labeled promastigotes. Microscopic examination of cytospin slides prepared from these sorted cells revealed that only $84.6\% \pm 5.1\%$ of CFSE-positive monocytes contained intracellular parasites visible by microscopy ($n = 3$ assays). We hypothesize that flow cytometry may be more accurate than microscopy as a measure of initial phagocytosis, since a low level of early parasite death may preclude microscopic detection of all ingested parasites.

Oxidant production by monocytes infected with live *Leishmania*. Promastigotes were labeled with CFSE prior to their phagocytosis by human peripheral blood monocytes. Monocytes were subsequently labeled with DHE (also called hydro-
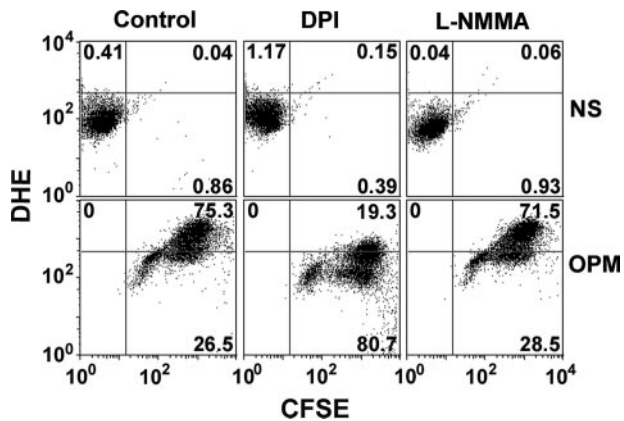
FIG. 4. Inhibition of the phagocyte oxidase suppresses DHE oxidation. MACS-purified monocytes were preincubated with either DPI, an inhibitor of the NADPH oxidase, or L-NMMA, an iNOS inhibitor. Cells were incubated with CFSE-labeled opsonized *L. chagasi* promastigotes (OPM) or buffer alone (NS). Oxidants were labeled with DHE after 10 min of infection, and cells were processed for flow cytometry after an additional 10 min. The basal level of oxidized DHE due to metabolic activity of control uninfected monocytes (NS) was used to set gates. Numbers indicate the percentages of total monocytes in quadrants. Data are representative of three separate experiments.

ethidine) to detect oxidants produced during phagocytosis. Cytosolic DHE emits blue fluorescence, but after the probe is oxidized to ethidium it intercalates into cellular DNA and emits red fluorescence. DHE is oxidized by intracellular hydrogen peroxide via either a peroxidase, cytochrome *c*, or Fe^{2+}. As DHE can scavenge reactive oxygen intermediates and falsely lower the oxidative response (37), we performed preliminary kinetic and concentration studies to determine the optimal time of DHE addition relative to the phagocytic stim-

ulus. The addition of DHE at least 5 min after phagocytosis and 10 min before fixation of cells for flow cytometry was optimal.

Background DHE oxidation occurred due to the metabolic activity of the monocytes, as observed in other studies (7, 37), and this was used to set the gates for flow cytometric assays. A further increase in DHE oxidation occurred after incubation with CFSE-labeled *L. chagasi* promastigotes under conditions favoring phagocytosis (Fig. 4). Most of the increase occurred in CFSE-positive monocytes that had phagocytosed *Leishmania*, suggesting that the response occurred preferentially in infected cells. To distinguish whether the increased DHE oxidation occurred due to an oxidative response or to increased metabolism induced by phagocytosis, inhibitors of inducible nitric oxide synthetase (iNOS) (NG-monomethyl-L-arginine [L-NMMA]) or of the NADPH oxidase (diphenylene iodonium [DPI]) were employed (8). Preincubation with the flavoprotein inhibitor DPI caused a fourfold reduction in the DHE oxidation caused by phagocytosis of opsonized promastigotes, whereas L-NMMA did not alter DHE oxidation. These data suggest that DHE oxidation reflected the respiratory burst due to the induction of NADPH oxidase activity in human monocytes.

Monocytes were infected with various ratios of parasites to monocytes (multiplicity of infection [MOI] of 1:1 to 5:1) (Fig. 5A). Accordingly, higher levels of infection were reflected by increasing CFSE stain intensity. Furthermore, higher infection levels led to increased DHE oxidation 20 min after phagocytosis, according to the intensity of the DHE stain. Also, stimulation with IFN-γ and/or LPS 24 h prior to assay resulted in detectable changes in the oxidant production by monocytes, as detected by DHE staining (Fig. 5B).

A representative flow cytometry plot (Fig. 6) illustrates the kinetics of DHE oxidation at 10 to 25 min after *Leishmania*
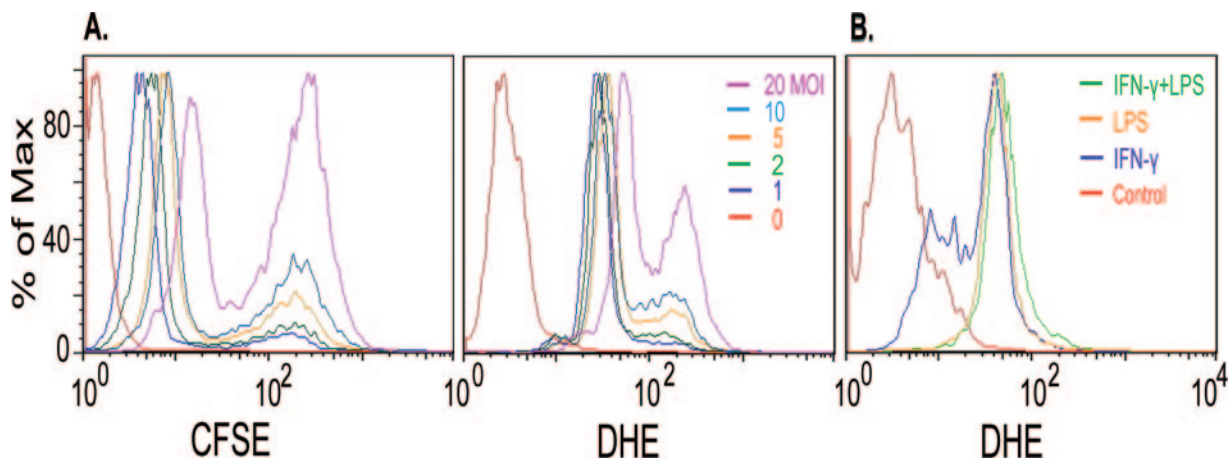

FIG. 5. (A) Representative histograms showing that the intensity of CFSE staining increases when monocytes are infected with opsonized CFSE-labeled promastigotes with increasing numbers of parasites (MOI) (left panel). Furthermore, the mean fluorescence intensity of the DHE stain increased in correspondence to the increased MOI (right panel). Cells were fixed for flow cytometry 20 min after phagocytosis. (B) Monocytes were exposed to buffer, IFN-γ, LPS, or IFN-γ plus LPS for 24 h and to DHE for 10 min prior to fixation for flow cytometry. This representative histogram shows mean fluorescence intensity corresponding to DHE oxidation. IFN-γ exposure resulted in two populations of cells with intermediate or high oxidized DHE staining. The increased DHE oxidation occurring after exposure to LPS was no greater than the DHE signal when IFN-γ-primed monocytes were stimulated with LPS. Data are representative of three repeat experiments.
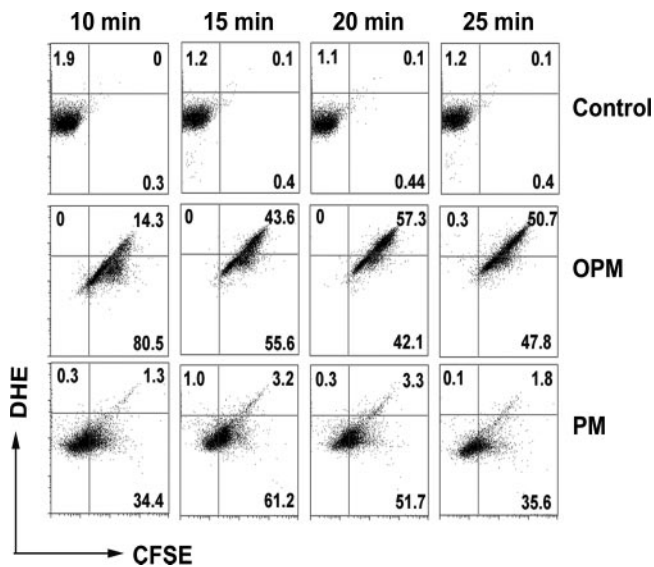
FIG. 6. Representative flow cytometry plot. MACS-purified human peripheral blood monocytes were infected with CFSE-labeled promastigotes, followed by DHE labeling to detect oxidants. The basal level of oxidized DHE due to metabolic activity of control uninfected monocytes was used to set gates. Monocytes were infected with CFSE-labeled promastigotes that were either opsonized with C5-deficient serum (OPM) or not opsonized (PM) for the indicated times. Numbers indicate the percentages of total cells in quadrants.

[automated magnetic cell sorting]; Miltenyi Biotec) purification, and parasites and dead cells were omitted by gating on live monocytes during flow cytometric analysis. The validity of the gates was verified by CD14 staining (not shown).

Several observations can be made. First, the respiratory response increased during the first 20 min after phagocytosis (Fig. 7A). Second, the amount of oxidant generated (Fig. 7A), as well as the proportion of infected cells that produced detectable oxidants (Fig. 7B), was higher in cells phagocytosing opsonized as opposed to control nonopsonized parasites ($P < 0.05$). Thus, the more vigorous DHE response in these cultures was not merely a reflection of higher infection levels. Third, most of the DHE oxidation occurred in cells that had taken up CFSE-positive live promastigotes (compare CFSE-negative and CFSE-positive bars in Fig. 7B), suggesting that the respiratory burst was not due to bystander monocytes or phagocytes exposed to parasite debris.

Although there may be some local opsonization of promastigotes at the macrophage/monocyte surface (47), it seems highly likely that promastigotes are opsonized by serum or interstitial components upon inoculation into a host. As such, the nonopsonized condition could be regarded as a control particle lacking the usual level of opsonization with complement and other host proteins.

Lack of TGF-β-mediated inhibition of the monocyte oxidative response. TGF-β has been reported to inhibit phorbol myristate acetate-induced oxidative responses of phagocytes in some cultured phagocytes (6, 44). To determine whether TGF-β inhibits the oxidative response of human cells to *Leishmania*, monocytes were preincubated with TGF-β for 16 h prior to infection with CFSE-labeled opsonized or nonopsonized promastigotes. TGF-β was unable to inhibit monocyte oxidative responses upon phagocytosis of nonopsonized or opsonized *L. chagasi* over a wide concentration range (Fig. 7C). Although there appeared to be moderate inhibition at 300 pg/ml, statistical analysis (ANOVA) detected no differences
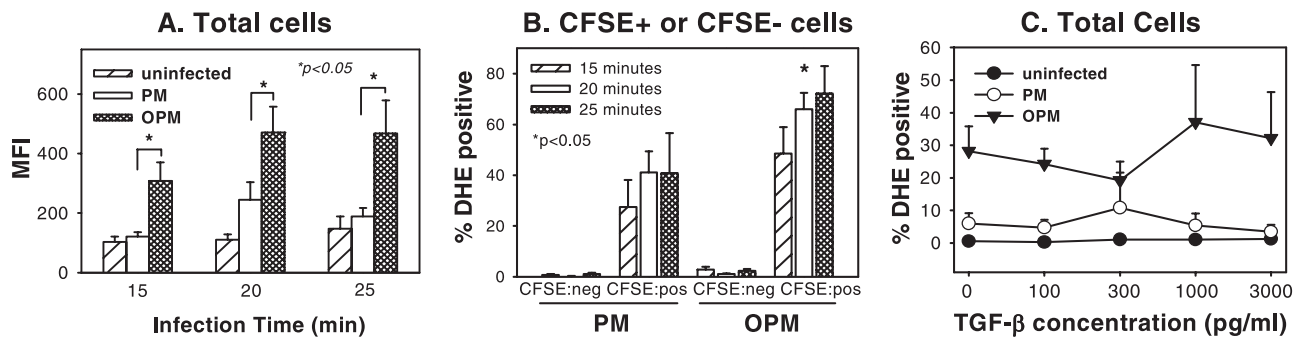
phagocytosis. Increased compensation did not alter the diagonal pattern in plots representing cultures infected with CFSE-labeled *Leishmania*. This could reflect the direct relationship between the number of parasites infecting a monocyte and the intensity of DHE oxidation as illustrated in Fig. 5A. The mean results from six independent experiments are shown in Fig. 7. Due to the requisite 10 min of DHE exposure to detect a respiratory burst, the earliest time point was 15 min. Non-monocytes were removed by monocyte MACS (autoMACS

FIG. 7. (A) The mean fluorescence intensity of monocyte cultures "infected" with buffer alone (uninfected), infected with opsonized promastigotes (OPM), or infected with nonopsonized promastigotes (PM) was determined at 15 to 25 min after infection was initiated. Bars show the mean fluorescence intensity (MFI) ± standard error from six separate experiments (statistical analysis was by ANOVA). (B) Within monocyte cultures infected with PM or OPM, cells that became CFSE positive (infected) or cells that remained CFSE negative (uninfected) were examined for whether they produced oxidants. Data show the percentage of DHE-positive infected monocytes [upper right /(upper + lower right)] × 100 or the percentage of DHE-positive uninfected monocytes [upper left/(upper + lower left)] × 100 in each culture. Bars represent the means and standard deviations from six separate experiments. (C) Lack of inhibitory effect of TGF-β on monocyte oxidant generation. Human monocytes were preincubated overnight with the indicated concentrations of TGF-β, after which they were infected with opsonized or nonopsonized CFSE-labeled promastigotes. Oxidants were detected 20 min later by DHE staining. The percentage of infected cells that underwent a respiratory burst was calculated from the events in the CFSE-DHE double-positive quadrant divided by the total cells times 100. Data represent the means and standard deviations from three experiments. Statistical analysis was done by one-way ANOVA.

between DHE responses in control and TGF-β-containing cultures at any concentration tested.

DISCUSSION

Classical activation of macrophages and monocytes by soluble or particulate stimuli results in assembly of the phagocyte NADPH oxidase with resultant production of superoxide (O_2^-). O_2^- coverts to H_2O_2 in a spontaneous or enzyme-mediated reaction, and in the presence of catalytic iron and H_2O_2 the microbicidal molecule hydroxyl radical (OH) is formed (8, 21). Particularly murine macrophages also express iNOS in response to activation, which catalyzes formation of the microbicidal oxidant nitric oxide (NO) (17). As documented in several studies, phagocytosis of *Leishmania* protozoa by human monocytes and macrophages in the presence of another signal such as IFN-γ can induce O_2^- production, resulting in intracellular parasite killing (27, 36). Studies of gene knockout mice have documented the importance of iNOS and the NADPH oxidase in control of murine *L. donovani* infection (26). Nonetheless, *Leishmania* infection itself can suppress oxidative and IFN-γ-mediated responses in cultured human or murine monocytes/macrophages, at least in part through activation of phosphatases, including SHP-1 (30, 32, 35). The necessity for the parasite to adequately suppress or evade oxidative responses in order to survive intracellularly led us to investigate a single-cell assay to detect the response of phagocytes to the internalization of individual live *Leishmania* promastigotes.

The visceralizing *Leishmania* spp. such as *L. chagasi* and *L. donovani* lose virulence quickly during in vitro cultivation. Thus, the 3 to 4 weeks required to raise clonal parasites expressing a fluorescent marker is sufficient time to lose the expression of important virulence factors (15). Therefore, we developed a method for short-term fluorescent labeling of *Leishmania* spp. in either an attenuated or virulent state and in either the promastigote or the amastigote stage.

The fluorescent markers tested were CFSE, CMTMR, and CM-DiI. CFSE spontaneously and irreversibly couples to available intracellular or surface protein amine groups such as lysine side chains. When cells divide, cytoplasmic CFSE label is distributed equally between daughter cells (18). CFSE has previously been used to study the response of human peripheral blood cells to infection with *Trypanosoma cruzi* in a flow cytometric assay (43). CMTMR is a fluorescent dye that diffuses through live cell membranes and reacts with intracellular glutathione *S*-transferase to produce a membrane-impermeant glutathione-fluorescent dye adduct. It has been used to document T-cell division and cell adhesion (42, 49). CM-DiI is a lipophilic cell membrane marker that is weakly fluorescent in water but highly fluorescent when it intercalates into membranes (1). The most efficient labeling of *L. chagasi* promastigotes was achieved with CFSE. CFSE-labeled promastigotes could be detected by flow cytometry, confocal microscopy, or time-lapse video microscopy.

The number of human monocytes infected with CFSE-labeled promastigotes was slightly higher when quantified by flow cytometry as opposed to microscopy. Based on video microscopy, we hypothesized that this occurred because a subset of parasites lyse within minutes of entering monocytes,

releasing dye and generating CFSE-positive monocytes without intact parasites. If this is the case, then flow cytometry may be more sensitive than microscopy for detecting the initial level of infection. Using short-term fluorescence labeling, we discerned that monocyte phagocytosis of opsonized promastigotes was more efficient than phagocytosis of nonopsonized promastigotes.

DHE (hydroethidine) has previously been used to analyze a phagocyte respiratory burst. Cytosolic dihydroethidium exhibits blue fluorescence, but once oxidized to ethidium it intercalates in DNA and stains the nucleus bright fluorescent red (38, 41). DHE is oxidized in resting leukocytes, likely through the uncoupling of mitochondrial oxidative phosphorylation, and this background was detected at a constant level in our flow cytometry assays. DHE is also oxidized by reactive species generated during phagocyte activation, including O_2^- H_2O_2, and NO. Although other methods may be more quantitative for detection of oxidant generation, this stain enabled flow cytometric evaluation of oxidant generation by promastigote-infected cells.

We applied these staining methods to detect individual monocytes that underwent a respiratory burst after phagocytosis of CFSE-labeled promastigotes. We observed DHE oxidation at above background levels soon after *Leishmania* phagocytosis by monocytes. Abrogation of this response with the specific phagocyte oxidase inhibitor DPI, but not with L-NMMA, suggested that DHE oxidation had resulted from activity of the NADPH oxidase rather than iNOS (8). Furthermore, a majority of monocytes infected with opsonized promastigotes underwent an oxidative burst and became DHE positive, whereas nonopsonized promastigotes activated only a subset of cells. TGF-β was an inefficient inhibitor of oxidant production, particularly at the high levels (1 ng) used in many TGF-β assays. The latter is particularly relevant to *L. chagasi* infection, since TGF-β is a determining factor in the murine adaptive immune response to *L. chagasi*, it is abundant in infected humans, and the invading parasite itself can activate latent TGF-β in tissues (11, 46). Apparently TGF-β does not directly promote parasite survival through suppressing oxidative responses of individual phagocytes.

Opsonized serum components enhance both the uptake and survival of *Leishmania* promastigotes in phagocytes. Opsonization with C3 and its fragment derivatives occurs after exposure to either serum or macrophages; the latter occurs through complement components locally produced and opsonized at the macrophage surface. Phagocytosis of promastigotes occurs through receptors for serum complement components such as CR3 and CR1 (3, 23, 25). Due to the low capacity of CR3 to simulate subsequent macrophage oxidative responses, uptake through CR3 could enhance parasite survival (48). It has been shown that opsonization with the third complement component facilitates uptake and intracellular survival of *L. major* in murine macrophages (24), whereas *L. major* survival in human neutrophils is adversely affected by serum opsonization (14). Finally, opsonization affects downstream intracellular signaling events and possibly the pathway of phagocytosis during *L. amazonensis* infection (22). These different roles for complement and complement receptors may vary depending on the source of phagocyte and *Leishmania* species. Our data suggest that, at least in human monocytes, opsonized serum compo-

nents actually enhance rather than suppress the generation of oxidants after *Leishmania* exposure. The fact that control non-opsonized parasites produced a lower-magnitude respiratory burst in infected cells illuminates a balance between greater entry into phagocytes and greater killing by phagocyte microbicidal processes, with which the parasite must contend when it enters a host cell.

The presence of intracellular *Leishmania* spp. suppresses macrophage major histocompatibility complex class I and II antigen expression, expression of inflammatory cytokines, calcium fluxes, and protein kinase C mobilization (32, 39). Cellular responses to gamma interferon are also suppressed (29), at least in part through activation of the phosphatase SHP-1 by the parasite protein EF-1α (28, 30). Here we report the ability of *L. chagasi* promastigotes to induce an early respiratory burst in human monocytes, particularly when opsonized with serum components. The responses of other cell types to promastigotes, and phagocyte responses to different stages or species of *Leishmania*, will be relevant and are feasible experiments using the short-term fluorescence labeling technique.

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