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Bacterium-Host Cell Interactions at the Cellular Level: Fluorescent Labeling of Bacteria and Analysis of Short-Term Bacterium-Phagocyte Interaction by Flow Cytometry

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Flow cytometry is a potentially powerful tool for analyzing the interactions of facultative intracellular bacteria and macrophages on a cellular level, particularly when fluorochromes are used to label the bacteria. We labeled *Listeria monocytogenes* and *Salmonella typhimurium* with a lipophilic dye, PKH-2, and used flow cytometry to investigate phagocytosis by J774A.1 cells and short-term bacterial survival. Labeled and unlabeled bacteria were identical in terms of viability, growth kinetics, and survival within macrophages, although recovery per macrophage was much greater for *L. monocytogenes* than for *S. typhimurium*. Using *L. monocytogenes* as a prototypical facultative intracellular bacterium, we estimated bacterial survival during phagocytosis on the basis of linear fluorescence measurements of infected J774A.1 cells and recovery of *L. monocytogenes* from sorted cells. The lower percentage of surviving *L. monocytogenes* in macrophages containing higher bacterial loads indicated the accumulation of nonviable bacteria within phagocytes. Removal of the external source of viable bacteria by washes and gentamicin treatment reduced the percentage of surviving intracellular *L. monocytogenes* to a baseline level, and all baseline levels were similar, regardless of bacterial load. *Listeria* enrichment recoveries, derived from individually sorted J774A.1 cells, demonstrated the heterogeneity of macrophages in intracellular bacterial survival, especially within heavily infected cells. These results indicated that survival of *L. monocytogenes* was dependent on the adaptations of a small fraction of bacteria within a population of macrophages which permit intracellular growth.

The relationship between pathogenic bacteria and host phagocytic cells is central to the induction of immunity. Certain organisms, including *Listeria monocytogenes* and *Salmonella typhimurium*, can survive within host macrophages. Circumvention of the immune response at this level allows subsequent bacterial growth, which ultimately produces disease in the infected individual. Alternatively, destruction of phagocytized bacteria provides a source of processed bacterial antigens against which T-cell- and antibody-mediated immune responses are directed. Pathogens of this nature are termed facultative intracellular bacteria (1, 3, 11, 14, 15).

The difficulty of quantifying bacterium-phagocyte interactions in vivo has led some investigators to use in vitro infection of macrophages to characterize bacteria as facultative intracellular bacteria, to determine the pathogenic potential of mutants screened in genetic studies, and to investigate the mechanisms by which facultative intracellular bacteria survive and grow within macrophages (9, 17, 18). Although in vitro systems provide both a quantitative readout and a means to control the level of macrophage infection, they are not well suited for monitoring the events (attachment, phagocytosis, and intracellular and extracellular killing) that occur between addition of bacteria and enumeration of surviving bacteria over time. Because in vitro infections are typically assayed by measuring bacterial recovery from serial dilutions of lysed, infected eucaryotic cell cultures, bacterial enumeration reflects the status of an entire population of macrophages, which can be highly variable (6). Therefore, bacterium-host cell interactions could be more clearly elucidated if studied at the cellular level.

Flow cytometry provides a potentially powerful tool for analyzing bacterium-host cell interactions, particularly if fluorophores which do not affect the viability of the bacteria or macrophages are used. Although fluorescent antibodies have been used to identify intracellular, attached, and freely suspended bacteria (4, 8), this approach may alter the viability or receptor properties of bacteria that are labeled before exposure to eucaryotic cells. Moreover, fluorescent-antibody binding is dependent on the availability of the epitope(s) and the stability of the antigen-antibody reaction. Direct labeling of bacteria with fluorescein derivatives can affect protein moieties on the bacterial surface; leaching of the dye is also a major concern. Nucleic acid-binding dyes Hoechst 33258, chromomycin A3, and acridine orange have been used to characterize bacterial species in a flow system (16, 27). Acridine orange has been used effectively to label bacteria in macrophage phagocytosis and HeLa cell invasion studies (16). These dyes, however, are toxic, making bacterial recovery impossible.

The lipophilic dye PKH-2 (Zynaxis Cell Sciences, Phoenixville, Pa.), which has been used to label mammalian cell membranes, is stable and nontoxic and does not affect the functional characteristics of the cells (12, 13). We established parameters for labeling bacteria with this fluorophore and used *L. monocytogenes* and *S. typhimurium* in conjunction with flow cytometry to study the uptake and short-term survival of facultative intracellular bacteria in the mouse macrophagelike cell line J774A.1.

MATERIALS AND METHODS

Bacteria. *S. typhimurium* SR11 was kindly provided by Richard Warren and Dennis J. Kopecko, Walter Reed Army Institute of Research, Washington, D.C. The virulent *L. mono-*

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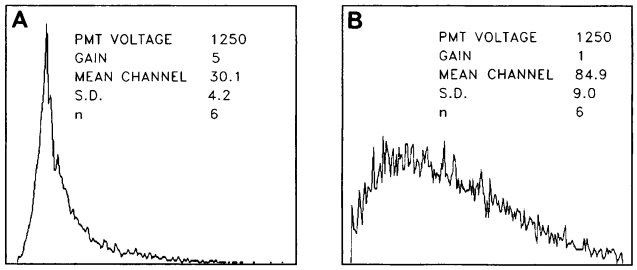

FIG. 1. Linear green fluorescence (GFL) histograms, representing *L. monocytogenes* (A) and *S. typhimurium* (B), immediately after labeling with PKH-2 (100,000 bacteria per histogram). Mean values from several experiments are shown in each panel, along with the flow cytometer settings used to generate these data. Abbreviations: PMT, photomultiplier tube; S.D., standard deviation.

cytogenes strain, EGD, was obtained from Robert North, Trudeau Institute, Saranac Lake, N.Y.

Cell culture. All tissue culture reagents were obtained from GIBCO Laboratories, Grand Island, N.Y. The macrophagelike cell line J774A.1 (21) was obtained from the American Type Culture Collection, Rockville, Md., and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μg of gentamicin per ml.

Fluorescent labeling of bacteria. A static 5-ml exponentially growing bacterial culture was pelleted, washed once in sterile phosphate-buffered saline (PBS), and resuspended in 0.5 ml of labeling buffer (Zynaxis). From this suspension, 50 μl was taken and mixed with 0.5 ml of labeling buffer in a polypropylene tube. PKH-2 dye (7.5 μl) was mixed with 0.5 ml of labeling buffer in another polypropylene tube. The contents of the two tubes were then mixed and incubated at room temperature for 10 min; the reaction was stopped by adding 10 ml of PBS containing 0.5% bovine serum albumin (PBS-BSA). The labeled bacteria were washed two times in 10 ml of PBS-BSA and resuspended in 0.5 ml of PBS-BSA. The concentration of bacterial suspensions was then estimated spectrophotometrically at 550 nm and adjusted to an A_{550} between 0.2 and 0.4. The actual number of bacteria per milliliter was determined by plating 10-fold serial dilutions of the suspension in duplicate. This number was used to calculate the multiplicity of infection (MOI) in all experiments.

Infection of macrophages. Monolayers of J774A.1 cells were washed three times by adding 20-ml portions of RPMI 1640 medium with no antibiotics to culture flasks, gently swirling the flasks, and decanting the medium. Cells were detached from the flask by vigorous agitation. Viable cells were counted with a hemacytometer and adjusted to a concentration of 5×10^5 cells per ml with antibiotic-free medium in a round-bottom polypropylene tube. These cell suspensions were allowed to equilibrate at 37°C in a 5% CO_2 atmosphere for 1 h before labeled bacteria were added. All assays were incubated statically at 37°C in a 5% CO_2 atmosphere. After incubation for 1 h, extracellular bacteria were removed from the assay system by two centrifugal washings (5 min at $100 \times g$) in RPMI 1640 medium with 10 μg of gentamicin (GIBCO) per ml, and resuspended to the original reaction volume in medium containing 10 μg of gentamicin per ml. This point was referred to as time zero ($T = 0$). In experiments monitoring bacterial uptake, test samples were taken at the described intervals leading up to time zero.

Flow cytometry. For all flow cytometry analyses, we used an EPICS CS flow cytometer (Coulter Cytometry, Hialeah, Fla.) equipped with an argon laser set at 500 mW and tuned to the excitation wavelength (488 nm) for PKH-2 dye. Infected viable cells fluoresced green under these conditions. Dead cells were observed by adding propidium iodide (final concentration, 0.05 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) to the samples. This dye binds to the DNA of dead cells, causing them to fluoresce red. Data for the following four parameters were collected and used for setting gates and windows on the cell sorter: (i) forward light scatter (FLS); (ii) log-amplified green fluorescence (LGFL); (iii) log-amplified red fluorescence (LRFL); and (iv) linear-amplified green fluorescence (GFL).

Debris, free bacteria, and cell clumps were excluded from analysis and sorting on the basis of FLS. The infected J774A.1 cell population was defined by a two-parameter LGFL-versus-LRFL histogram, which allowed discrimination of low and high green fluorescence intensity populations and live versus dead phagocytes. The specific population to be sorted was selected by drawing a bitmap graphic around the cells of interest. Some experiments involved sorting on the basis of channel windows from GFL histograms, which were derived by using the entire viable cell population, as defined by a bitmap graphic, and FLS. With the instrument settings optimized for J774A.1 cells, FLS signals of free bacteria were undetectable; therefore, for

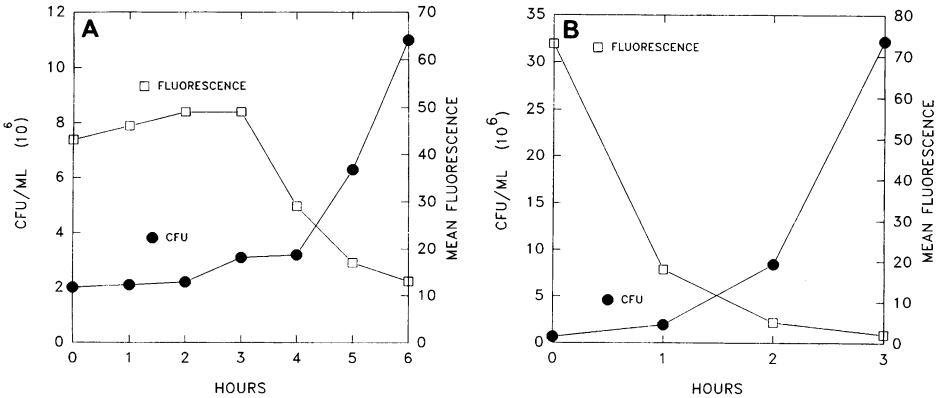

FIG. 2. Effects of bacterial replication on the fluorescence intensity (mean peak channel) of PKH-2-labeled *L. monocytogenes* (A) and *S. typhimurium* (B). Aliquots were taken from bacterial cultures immediately after labeling ($T = 0$) and hourly for GFL intensity analysis (10,000 bacteria per datum point). Recovery was determined by plating 10-fold dilutions of the culture on triplicate TSYEA plates.

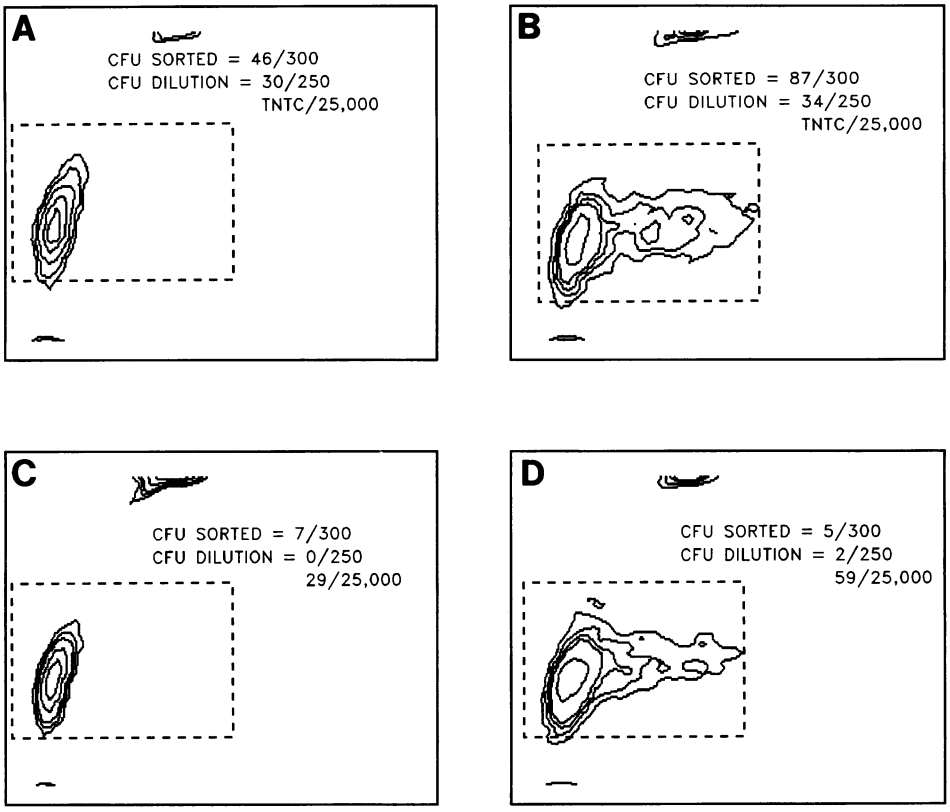

FIG. 3. Effects of PKH-2 labeling and flow cytometry procedures on the uptake and short-term recovery of *L. monocytogenes* (A and B) and *S. typhimurium* (C and D) from J774A.1 macrophages. Unlabeled (A and C) and PKH-2-labeled bacteria (B and D) are shown. The *x* axis represents LGFL, and the *y* axis represents LRFL. Each histogram represents 5,000 viable macrophages. CFU SORTED shows the number of bacteria recovered from 300 J774A.1 cells, which were sorted from within the bitmap represented by the dashed box. CFU DILUTION shows the number of bacteria recovered from serial dilutions of the indicated number of macrophages. Nonviable J774A.1 cells, stained with propidium iodide, are shown as the upper population in each panel. TNTC, too numerous to count.

measurements of labeled bacteria alone, we increased the sensitivity of the FLS detector by decreasing the neutral density filtration by a factor of 100.

Viability of bacteria. The viability of labeled *L. monocytogenes* was estimated by flow cytometry. The number of fluorescent bacteria (total count) present in a 50-μl sample was determined and correlated with viable plate counts. The number of total bacteria was normalized to account for instrument count rate averaging, which was based on comparisons of microscopic and instrument counts of 1-μm-diameter fluorescent microspheres (Polysciences, Warrington, Pa.).

Sorting of infected phagocytes and recovery of bacteria. Before starting each experiment, we sorted fluorescent beads (Coulter) and verified the accuracy of the sort microscopically. The accuracy of single-cell sorting into 96-well plates was also verified with beads. Replicates of the required number of cells were sorted into the wells of sterile 96-well plates (Costar Corp., Cambridge, Mass.) (each well containing 100 μl of sterile distilled water) with an Autoclone device (Coulter). A brief 5-min incubation period allowed the cells to become osmotically fragile, setting the stage for both enrichment and direct plating cultural recovery schema.

For enrichment recovery, 100 μl of 2× tryptic soy plus 0.6% yeast extract (Oxoid Ltd., Basingstoke, United Kingdom) enrichment broth (TSYEB) was added to each inoculated well. These broth cultures were monitored for up to 72 h for growth at 37°C. The number of infected viable eucaryotic cells containing viable intracellular bacteria was determined from these data for each channel range at each kinetic time point of an assay.

For direct plating recovery, each replicate was transferred to a sterile 1.5-ml microcentrifuge tube containing an additional 500 μl of sterile water. Glass beads (0.45 to 0.52 mm in diameter) (Thomas Scientific Co., Swedesboro, N.J.) were added aseptically, and each replicate was vortexed vigorously before plating. Mechanical and osmotic lysis of eucaryotic cells was confirmed by microscopic examination. The plating medium was TSYE plus 1.5% agar (TSYEA). In some experiments, 250 cells were sorted directly into each sterile 1.5-ml microcentrifuge tube containing 500 μl of sterile water.

Bacteria within J774A.1 cells were counted directly on cytocentrifuge spreads made from sorting 200 to 400 cells into 100 μl of RPMI 1640 medium containing 10% heat-inactivated fetal calf serum. The slides were examined directly by fluorescence microscopy or treated with Wright-Giemsa stain for standard microscopy.

RESULTS

Effects of labeling on bacteria. Optimal conditions for labeling bacteria with PKH-2 (uniformity and intensity of dye binding) were determined microscopically from a matrix titration. These conditions were similar to those that produced maximal labeling of eucaryotic cells (12). Fluorescence inten-

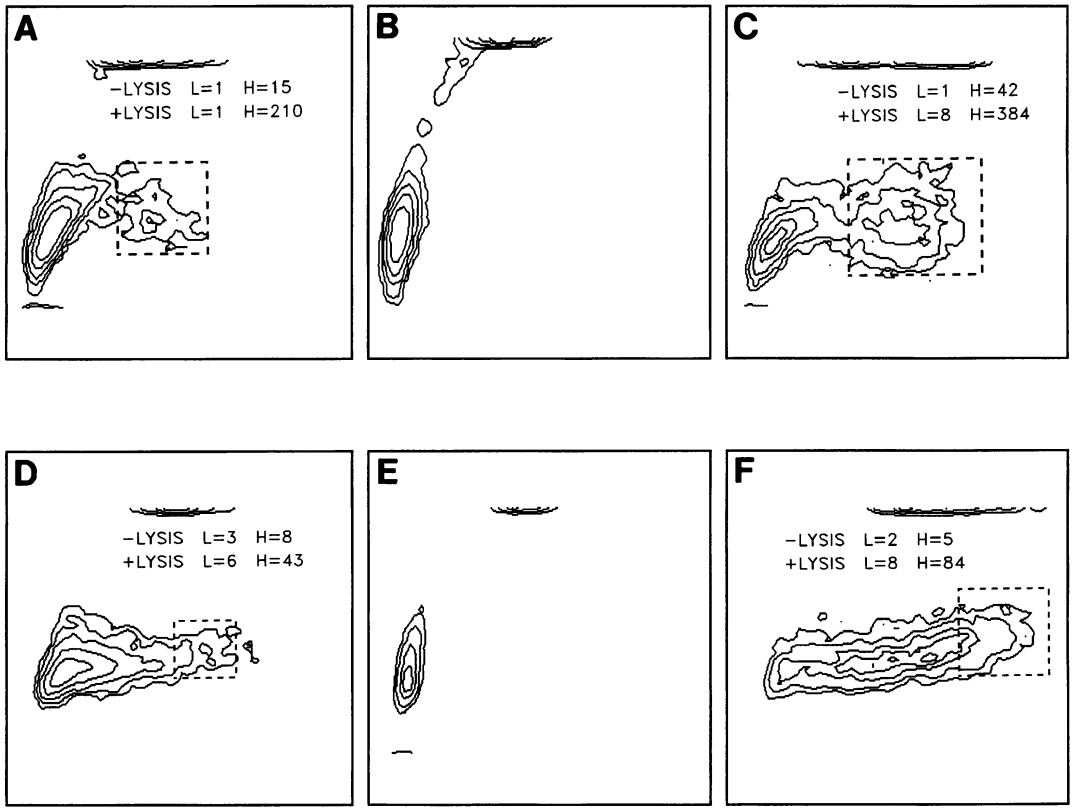

FIG. 4. Comparison of *L. monocytogenes* (A and C) and *S. typhimurium* (D and F) infection of J774A.1 cells at different MOIs. The MOIs for *L. monocytogenes* are 0.7 (A) and 7.0 (C), and the MOIs for *S. typhimurium* are 2.6 (D) and 13.0 (F). Autofluorescence of uninfected J774A.1 cells is shown (B and E). The *x* axis represents LGFL; the *y* axis represents LRFL. Each histogram represents 5,000 viable macrophages. Osmotic and mechanical disruption (+LYSIS) was used to release intracellular bacteria from macrophages; values with no lysis treatment (−LYSIS) are shown. H and L refer to the numbers of high and low LGFL intensity populations of infected macrophages, respectively. Bacteria were recovered from replicate sorting of 300 J774A.1 cells for H and L populations. The dashed boxes outline the bitmaps used for sorting the H population. The mean LGFL intensities for these boxes are 19.1 (A), 36.4 (C), 73.9 (D), and 249.0 (F).

sity of bacteria was consistent: labeled *S. typhimurium* cells were approximately 14 times brighter, with a broader range of fluorescence intensities, than were labeled *L. monocytogenes* cells (Fig. 1). Flow cytometry measurements were made before the start of each infection experiment to ensure comparability of results.

To determine the effects of labeling on bacterial viability, labeled and unlabeled *L. monocytogenes* and *S. typhimurium* were standardized spectrophotometrically and compared on the basis of recovery on agar and growth kinetics. The results for labeled and unlabeled bacteria were identical, indicating a nontoxic effect by PKH-2. The viability of labeled bacterial cultures was routinely 70 to 95%.

The effect of cell division on fluorescence of labeled bacteria was determined on cultures of labeled *L. monocytogenes* and *S. typhimurium*. A linear, inverse correlation between bacterial growth and fluorescence was observed (Fig. 2). The stability of labeling was demonstrated by the fact that the fluorescence intensity of labeled *L. monocytogenes* remained constant during lag phase, whereas replication resulted in a 50% decrease in fluorescence with each cell division.

In vitro infection of phagocytes with PKH-2-labeled bacteria. The values for survival of labeled and unlabeled *L. monocytogenes* and *S. typhimurium* in macrophages were examined with and without cell sorting to assess the effects of PKH-2 labeling and flow cytometry on bacterial uptake and intracellular viability. Bacterial recovery was determined at time zero (1-h phagocytosis reaction; extracellular washing step), either by a standard dilution method or cell sorting (Fig. 3). In the dilution method, infected macrophages were lysed and plated on agar at 10-fold dilutions. In the flow cytometry method, the entire macrophage population containing either labeled or unlabeled bacteria was included within a bitmap sorting region, and replicates of 300 viable cells were sorted (Fig. 3). The values for recovery of *L. monocytogenes* and *S. typhimurium* were equivalent for labeled and unlabeled organisms. Similar numbers of viable organisms were present in 300 sorted cells or an approximately equal number not processed by flow cytometry. Data for a larger number of macrophages also provided a reference point for the numbers of cells typically used in standard dilution methods (4, 5, 9, 20). In all cases, recovery of *S. typhimurium* was lower than that of *L. monocytogenes*.

Even though the MOI and fluorescence distributions were similar for both test organisms, the actual level of infection in J774A.1 cells was presumed to be greater for *L. monocytogenes* because of the higher mean fluorescence intensity per organism of *S. typhimurium* (Fig. 1). To address this issue, J774A.1 cells infected with labeled *L. monocytogenes* or *S. typhimurium* at high and low MOIs were sorted on the basis of fluorescence

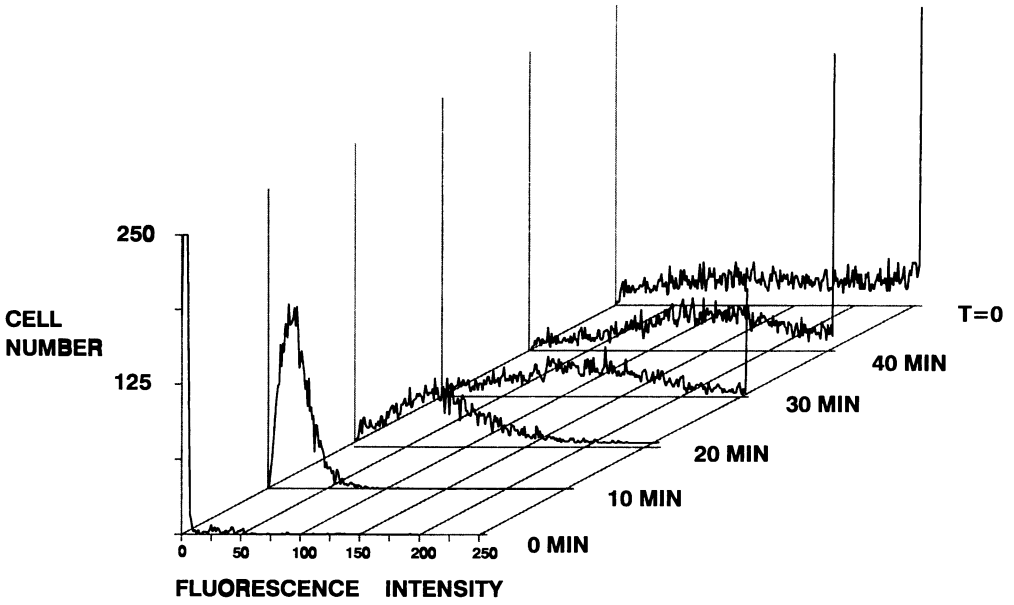

FIG. 5. Uptake of PKH-2-labeled *L. monocytogenes* by J774A.1 macrophages monitored by flow cytometry. The *x* axis represents GFL; the *y* axis represents cell number. Times following the addition of bacteria are shown. After 1 h, a portion of the original infected macrophage suspension was washed by centrifugation and resuspended in medium containing gentamicin ($T = 0$). Autofluorescence of uninfected J774A.1 cells (0 min) is also shown. Each histogram represents 5,000 viable macrophages.

intensity. The different MOIs were reflected in the fluorescence distribution of the infected macrophages (Fig. 4), i.e., the higher MOI produced a greater increase in fluorescence intensity for both organisms. The number of viable bacteria recovered was proportional to the fluorescence intensity of the infected macrophages. The recovery of *S. typhimurium* from the most heavily infected J774A.1 cells, however, was markedly lower than that of *L. monocytogenes*, even though the mean fluorescence of the brightest sorted cells (in box) was approximately 10 times higher. This observation suggested that the lower recovery of *S. typhimurium* resulted from fewer viable bacteria, rather than a lower average number of bacteria per macrophage.

Extracellular quenching of PKH-2 by the addition of crystal violet (16) established that labeled bacteria were indeed intracellular (data not shown). The increased numbers of bacterial colonies after macrophage lysis, compared with the number of colonies from intact macrophages, was also consistent with the intracellular location of bacteria, as was the increased difference between lysed and nonlysed cells in the population with a higher fluorescent intensity.

Quantitation of bacterial phagocytosis and killing. The histograms produced by log-amplified fluorescence signals established the basic parameters of the assay by enhancing discrimination between populations of heavily infected macrophages (H) and macrophages with few or no bacteria (L) (Fig. 4). In subsequent experiments, linear-amplified signals were used for more precise quantitation of intracellular bacteria, because the channel number is directly proportional to fluorescence intensity and, therefore, to the number of bacteria present within a macrophage. *L. monocytogenes* was used in these experiments because (i) our initial experiments indicated that it was more readily recovered from J774A.1 cells than *S. typhimurium* was, (ii) it has been studied extensively as a model facultative intracellular bacterium (2, 3, 6, 11, 23), and (iii) the fluorescence per organism was less varied (Fig. 1).

Macrophage phagocytosis and killing of labeled *L. monocytogenes* were examined in a series of experiments. The changes in infected J774A.1 cell fluorescence (linear-amplified signals) during a typical in vitro phagocytosis experiment (Fig. 5) showed an overall increase between 0 and 40 min, resulting in a broad distribution across the 256 fluorescence channels at time zero ($T = 0$). J774A.1 cells infected with *L. monocytogenes* were sorted by a series of fluorescence windows (channel ranges). Three types of data were collected from sorted cells: (i) agar plate counts to determine the average number of viable bacteria from 100 viable J774A.1 cells; (ii) single-cell enrichment cultures, produced by sorting one viable macrophage into each well of a 96-well cluster, to determine the number of J774A.1 cells containing at least one viable bacterium; and (iii) microscopic counts of cytocentrifuge slides to enumerate fluorescent or Giemsa-stained bacteria within cells.

Direct relationships between increased fluorescence and both the numbers of cells containing at least one viable bacterium and the average number of viable bacteria per macrophage were observed (Table 1). At no point were 100% of the sorted cells positive for viable bacteria, even though the average number of bacteria recovered per cell was approximately one in the most fluorescent population. Observed microscopically, particulate fluorescent material (labeled bacteria or bacterial debris) was present in increasing amounts in every macrophage of all windows except channels 1 to 5, which coincided with the autofluorescence peak of uninfected J774A.1 cells. The plating recoveries of several windows, however, indicated that very few J774A.1 cells contained viable intracellular *L. monocytogenes.*

To further address the question of intracellular bacterial killing efficiency, the number of microscopically identifiable Giemsa-stained intracellular bacteria from each fluorescence window was compared with that of viable bacteria. The mean fluorescence intensity of labeled *L. monocytogenes* was determined by using the same flow cytometer settings as those used

TABLE 1. Correlation of fluorescence intensity with recovery of *L. monocytogenes* from J774A.1 macrophages and microscopically observed intracellular fluorescent material[a]

Fluorescence channels (GFL)	No. of CFU/ macrophage	No. of positive by wells, single-cell enrich-ment/96 wells	Discrete no. of par-ticles/mac-rophage
1–5	0.01	4	0.3
10–15	0.08	12	1.8
20–25	0.15	26	2.8
40–45	0.35	43	2.7
80–85	0.70	67	4.0
160–165	0.97	60	5.6
250–254	0.99	68	>10

[a] All measurements taken at time zero.

for macrophages infected with bacteria. These data and the mean fluorescence of uninfected J774A.1 cells were used to calculate the approximate number of phagocytized bacteria, which would account for the fluorescence of each window (Table 2). The accuracy of this approach was confirmed by microscopic counts of fluorescent microspheres within J774A.1 cells sorted from fluorescence windows. The microspheres remained intact within the macrophages, and their numbers correlated with the predicted number based on flow cytometry measurements (Table 2). For *L. monocytogenes*, this estimate exceeded the number of stained and recoverable bacteria, suggesting that most bacteria phagocytized during the 1-h incubation had been killed or destroyed. Bacterial debris was seen in many of the macrophages sorted from the higher channels. The high viability of labeled bacterial cultures made it unlikely that nonviable organisms in the inoculum contributed significantly to this effect.

The preceding experiments indicated significant killing of *L. monocytogenes* at time zero. Bacterial survival before this point was assessed by determining recovery of *L. monocytogenes* from cells sorted from fluorescence windows during the phagocytosis reaction. After the 60-min infection, half of the test sample was washed and resuspended in gentamicin as usual (time zero), and incubation was continued on the other half while washing took place (80-min elapsed time). As illustrated in Fig. 5, the numbers of cells for sorting were insufficient in some of the channel ranges, especially at 10 and 30 min (Table 3). Although the absolute number of *L. monocytogenes* per cell increased in proportion to fluorescence, the percentage of viable bacteria, based on the estimated bacterial load (fluorescent bacterial equivalents), decreased as fluorescence increased (Table 3). This result was consistent with the accumulation of killed bacteria. Similarly, recovery values remained relatively constant for a given window at 10, 30, 60, and 80 min; however, washing and treatment with gentamicin (time zero) removed the source of viable bacteria, resulting in a markedly lower bacterial recovery than that from time-matched unwashed test samples (80 min). This effect was more pronounced in the lower-fluorescence channels, so that all of the channel windows were equivalent with respect to bacterial viability at time zero.

DISCUSSION

We used fluorescent bacteria and flow cytometry to analyze bacterium-phagocyte interactions, which are important in the development of immunity and in the ability of a pathogen to survive in the host (1, 2, 3, 9). Labeling with PKH-2 was stable and nontoxic. Parameters such as viability, growth rate, and survival within phagocytes for labeled and unlabeled bacteria were indistinguishable. Intracellular bacterial recovery experiments comparing the cell sorting method with a standard recovery procedure (log dilutions of lysed eucaryotic cells) gave similar values; however, these experiments illustrated the difference in scale between the two methods when applied to *S. typhimurium* and *L. monocytogenes*. One hundred times the number of *Salmonella*-infected macrophages were required to produce an equivalent recovery. Recovery of *S. typhimurium* was consistently lower than that of *L. monocytogenes* even when we compensated for differences in the level of infection by selecting the most heavily infected phagocytes for sorting and recovery. This result suggested that a large portion of the *Salmonella* inoculum was killed by J774A.1 cells and is similar to findings of other studies, using standard in vitro infection and recovery methods, in which J774A.1 cells produced a 1- to 2-log-unit reduction in viable *S. typhimurium* during the first hour after infection (4).

Several observations point to significant killing of *L. monocytogenes* by J774A.1 cells. At all levels of infection, a significant proportion of phagocytes in single-cell enrichment cultures contained no viable *L. monocytogenes*. In addition, although bacterial recovery was proportional to the fluorescence of infected macrophages, neither the absolute number of recoverable bacteria nor the number of Giemsa-stained bacteria could account for the amount of fluorescent material present, particularly in the higher-fluorescence channels. Dividing the number of recoverable *L. monocytogenes* by the total

TABLE 2. Comparison of recoverable *L. monocytogenes* with microscopically enumerated bacteria and with predicted numbers of bacteria estimated by flow cytometry[a]

Fluorescence channels (GFL)	No. of CFU/cell[b]	No. of stained bacteria/cell	Estimated no. of bacteria (FBE)[c]	No. of beads/cell	
				Counted	Estimated
1–5	0.0	0.05	0–1	0	0
10–15	0.02	0.31	2	0	0
20–25	0.08	0.57	5	1.0	1
40–45	0.11	0.96	11	1.9	2
80–85	0.34	1.6	22	3.2	3
160–165	0.80	3.1	44	6.6	7
250–254	0.94	5.0	70	ND[d]	ND

[a] Similar data from J774A.1 cells which phagocytized 1-μm-diameter fluorescent beads are also shown.
[b] All measurements were taken at immediately after infection of J774A.1 cells (time zero) and are based on three replicates of 100 sorted J774A.1 cells.
[c] FBE, fluorescent bacterial equivalents (number of bacteria per macrophage, estimated by flow cytometry).
[d] ND, not determined.

TABLE 3. Survival based on infection level of *Listeria monocytogenes* in J774A.1 cells during a 1-h phagocytosis reaction

Fluorescence channels (FBE)[a]	No. of *L. monocytogenes*/J774A.1 cell[b] (% viable)[c]				
	During phagocytosis (min)				Before phagocytosis ($T = 0$)
	10	30	60	80	
1–5 (0–1)	0.16	0.13	0.13	0.14	0.01
10–15 (2)	0.36 (18.0)	0.47 (23.5)	0.38 (19.0)	0.43 (21.5)	0.05 (2.5)
20–25 (5)	–[d]	0.74 (15.8)	0.53 (10.6)	0.56 (11.2)	0.05 (1.0)
40–45 (11)	–	0.81 (7.4)	0.58 (5.3)	0.72 (6.5)	0.22 (2.0)
80–85 (22)	–	–	0.93 (4.2)	1.03 (4.7)	0.37 (1.7)
160–165 (44)	–	–	–	1.64 (3.7)	0.88 (2.0)

[a] FBE, fluorescent bacterial equivalents (number of bacteria per macrophage, estimated by flow cytometry).
[b] Based on plating recoveries from 100 sorted cells (triplicate counts).
[c] (Number of bacteria recovered per J774A.1 cell/FBE) × 100.
[d] –, insufficient number of cells for sorting.

number of bacteria phagocytized, as estimated by fluorescence (Table 2), resulted in viability estimates between 1 and 2%.

These results, which indicate significant bacterial killing, conflict with published reports that J774A.1 cells permit intracellular growth of virulent *L. monocytogenes* (7, 10, 20, 25). Two factors may explain this apparent discrepancy. First, the numbers of viable macrophages used in other studies were only approximate and were usually several orders of magnitude higher than those we obtained by cell sorting. Second, the total number of bacteria phagocytized over time could not be determined in those studies as the use of labeled bacteria and flow cytometry permitted in ours. Without this information, recovery of large numbers of bacteria would reasonably lead to the conclusion that J774A.1 cells are poorly bactericidal. This conclusion would be reinforced by increased subsequent recovery of bacteria, which occurs with *L. monocytogenes* (2, 5, 10, 20, 22, 25, 26).

Our studies also showed significant numbers of viable intracellular *L. monocytogenes* at time zero, especially compared with *S. typhimurium*. In additional experiments using the flow cytometry procedure, recovery increased at 6 and 24 h (22). The use of flow cytometry permitted fluorescence quantitation of the infection and the separation of precise numbers of viable cells. These capabilities, rather than the bacterial recovery data, indicate that most, but not all, bacteria are killed during the incubation phase of phagocytosis and imply that surviving bacteria, which subsequently divide and spread from cell to cell (7, 10, 22, 25, 26), have undergone adaptations for intracellular survival.

Intracellular growth of *L. monocytogenes* involves induction of listeriolysin (2, 24), a sulfhydryl-dependent hemolysin that lyses the endosome, giving the bacteria access to the cytoplasm (2, 5). Dabiri et al. (7), using two-color immunofluorescence and a microscopic image analysis system, determined that about 40% of immunologically detectable *L. monocytogenes* in J774A.1 cells were associated with actin after 60 min of incubation. Many of these viable bacteria may have escaped the phagolysosome of J774A.1 cells. In our system, if the number of Giemsa-stained bacteria, rather than total bacterial fluorescence, is used to calculate *Listeria* viability at time zero, the percentage increases from 2 to approximately 20. These approaches, however, do not account for bacteria that may have been degraded beyond morphological or immunological recognition, which appear to account for a large proportion of the fluorescent material present within heavily infected J774A.1 cells.

Bacterial viability determined during the 1-h incubation indicated that rapid phagocytosis and killing of *L. monocytogenes* by J774A.1 cells may account for the accumulation of nonviable bacteria within phagocytes. The relatively constant recovery for a given fluorescence window over time reflects rapid bacterial uptake by J774A.1 cells, with each time point representing a new set of macrophages containing a similar number of recently phagocytized *L. monocytogenes*. The percentage of viable *L. monocytogenes* associated with increased macrophage-bacterium fluorescence declines, because the proportion of recently phagocytized bacteria is lower in higher-fluorescence channel windows. We found that this situation persisted until the cells were washed and treated with gentamicin, which eliminated the external source of viable bacteria; the percentage of recovery then became constant at greatly reduced levels for all fluorescence windows, suggesting that a fixed fraction of bacteria adapt to the intracellular environment and that the higher levels of recovery during phagocytosis reflect bacteria recently taken up by macrophages but not yet killed.

Knowledge of bacterial viability within antigen-processing cells is potentially important for antigen presentation, since two distinct mechanisms of presentation by class I major histocompatibility complex molecules have been described (19). Bacterial antigens that escape the vacuolar system, which is the case for virulent *L. monocytogenes* (2, 3), have access to newly produced class I molecules in the cytoplasm. Antigens that remain in vacuolar compartments may be regurgitated and may access cell surface class I molecules. The mechanisms by which these events occur have not been characterized but are clearly relevant to the development of host resistance.

The flow cytometry system described here should prove to be useful in additional applications, such as studies involving mixed cell types that can be distinguished on the basis of light scatter or immunofluorescence staining. Studies of the effects of bacterial infection on surface moieties or other eucaryotic cell properties would be enhanced by the abilities to identify and quantitate the level of infection. Cell sorting allows for separation and manipulation of infected cells. These applications also extend to in vivo studies, where PKH-2 has been used extensively (12).

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