The intracellular killing of microorganisms in phagocytes involves the fusion of lysosomes containing bacterial factors with phagosomes, and several intracellular pathogens are able to inhibit this fusion event. In this study, we report the reconstitution of phagosome-lysosome fusion in vitro, using an assay based on resonance energy transfer between fluorescent phospholipid analogues that were inserted into whole human NB4-neutrophil membranes from liposomes containing positively charged lipids. Cytosol was required for fusion, and fusion was stimulated 3-fold if this cytosol had been prepared from neutrophils activated by using opsonized zymosan or a combination of the calcium ionophore (A23187) and phorbol myristate acetate (PMA). Fusion was inhibited by the addition of PP1, an inhibitor of Src family protein kinases, or GTPγS. We have previously reported that the biogenesis of phagolysosomes in human neutrophils is inhibited by mycobacteria. Here we show that cytosol from cells having internalized live (not heat-killed) Mycobacterium smegmatis or cytosol simply incubated with mycobacteria inhibited fusion, indicating that soluble factors are involved in mycobacterial inhibition of phagosome-lysosome fusion.

Neutrophils, monocytes, and macrophages take up bacteria by phagocytosis. Phagosomes fuse with endosomal and lysosomal compartments, delivering enzymes, bactericidal proteins, and proton pumps to the phagosomes (1). These factors are thought to play a major role in the intracellular killing of microorganisms (2). Contributing to the intracellular survival of mycobacteria, phagosomes containing ingested live mycobacteria do not reach a pH lower than 6.3, as they do not acquire the lysosomal proton pump (3) and do not fuse with lysosomes while continuing to fuse with endosomes (4–7). Little is known about the mechanism of phagosome-lysosome fusion and its inhibition by mycobacteria, although some of the proteins involved in these processes have been identified (8–10).

We have previously shown that in human neutrophils, fusion between phagosomes and azurophil granules (specialized lysosomes) does not occur during infection with mycobacteria (11). Upon phagocytosis of zymosan by human neutrophils or macrophages, followed by phagosome-lysosome fusion, a tyrosine kinase of the Src family, Hck, was found to be activated on lysosomes and translocated to phagosomes (12–14). Translocation and activation did not take place during phagocytosis of mycobacteria, suggesting a role for this protein in regulating the biogenesis of phagolysosomes (11).

The major experimental problem in studying the mechanism of phagosome-lysosome fusion is the lack of an efficient in vitro assay for fusion (15). Moreover, it is difficult to purify lysosomes from macrophages, lacking a specific marker for this compartment. In this study, we took advantage of the fact that in neutrophils, lysosomes have specific markers such as myeloperoxidase and a high density and are thus easy to isolate by fractionation of the postnuclear supernatant on a discontinuous Percoll gradient (16). However, we have previously shown that centrifugation through Percoll removes peripheral proteins from the surface of vesicles (12, 17). Differential centrifugation avoids this problem, but neutrophils contain other types of granules that could potentially contaminate the lysosome preparation. Therefore, we have used NB4 cells that contain only one type of granule, the azurophil granule/lysosome (18). These promyeolytic human cells were differentiated to neutrophil-like cells by means of all-trans retinoic acid (14, 19). We developed a method to label NB4 cells with the fluorescent probes N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine (N-Rhodamine-PE) and N-(7-nitro-2,1,3-benzoazoxadiazol-4-yl) phosphatidylethanolamine (N-NBD-PE). We then measured fusion between labeled lysosomes that were isolated from these cells and unlabeled phagosomes.
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Lyosomal pellet was produced from a postnuclear supernatant by centrifugation at 12,000 × g for 15 min (14). Phagosomes containing 3-μm latex beads (PolySciences, Eppelheim, Germany), which had been incubated overnight at 30 °C with human serum from healthy volunteers to increase the extent of phagocytosis, were produced by the method of Desjardins et al. (1). Briefly, cells (5 × 10⁵/ml in MEM/HEPES) were incubated at a ratio of 15 beads per cell for 90 min at 37 °C, resuspended in 2 ml of sucrose 250 mm, 10 mM HEPES pH 7.4, and cavitated at 350 psi for 10 min. After cavitation, sucrose was added to the postnuclear supernatant to 40% w/w and loaded between the 62 and 35% layers of a 62:40:35:25:10% (w/w) sucrose gradient. After centrifugation in a SW41 rotor (Beckman) at 100,000 × g for 1 h, latex bead-containing phagosomes were recovered from the 10 to 25% interface (1). Sucrose was then removed from the phagosomes by pelleting the phagosomes at 50,000 × g for 15 min at 4 °C, in 10 ml of phosphate-buffered saline and resuspended in fusion buffer as described in the legend to Fig. 3. For thin layer chromatography, lipids were extracted according to Folch et al. (20) and run on silica gel plates using chloroform/methanol/water (60:25:4) as a solvent. Spots were visualized by excitation with broad spectrum UV light.

Fluorescence Measurements—Fusion was measured using a Photon Technologies International (PTI, South Brunswick, NJ) or SAFAS fix (Monte Carlo, Monaco) fluorometer, with continuous stirring in a thermostated cuvette at excitation and emission wavelengths of 465 and 530 nm, respectively. For calibration of the fluorescence scale, the initial residual fluorescence intensity was set to zero and the intensity at infinite probe solution 100%. The latter value was obtained after lysis of the liposomes with Triton X-100 (0.5% v/v) with correction for the quenching of NBD by this detergent (21), as described before (22). To prepare cytosol, a postnuclear supernatant of cells fractionated as described for lyosome preparation was centrifuged at 100,000 × g, and the supernatant was used. Spectra were obtained by excitation at 465 nm, using 2-nm slits throughout, on the PTI fluorimeter.

RESULTS

The phospholipid analogues N-NBD-PE and N-Rhodamine-PE are widely used to quantify membrane fusion with liposomes by a resonance energy transfer assay (21), because they do not spontaneously exchange between membranes by lipid transfer, in contrast to other probes that may give false positive results (23). However, no efficient method existed for introducing these probes into biological membranes. In order to incorporate them into the membranes of live cells, we produced dry lipid films consisting of N-NBD-PE, N-Rhodamine-PE, and the positively charged lipid DOTAP (2:2:1 molar ratio) and added pure water, producing liposomes as described under “Experimental Procedures.” DOTAP is widely used to transfect cells because it mixes with the lipids of cellular membranes, delivering the DNA into the cell (24). After 15 min of incubation of NB4 cells with these liposomes at 4 °C, we found that cellular plasma membranes were labeled by the fluorescent probes (Fig. 1a). After 15 min of incubation at 37 °C, followed by a chase in the absence of liposomes for 1 h at 37 °C, we found that intracellular vesicles were labeled, most likely by endocytosis of plasma membrane material (Fig. 1, b and c). Fluorescent NB4-neutrophils retained their capacity to interact with bacterial responses, as demonstrated by measuring superoxide generated by NADPH oxidase from cells stimulated by the calcium ionophore A23187 and PMA (results not shown). To isolate fluorescent lysosomes, cells labeled using a pulse-chase protocol were activated by incubation for 10 min at 37 °C in the presence of A23187 and PMA according to Welch and Maridonneau-Parini (14), a treatment that activates NADPH oxidase and stimulates lysosome exocytosis. Subsequently, the cells were lysed by nitrogen cavitation as described under “Experimental Procedures,” and the fluorescent lysosomes were isolated by differential centrifugation (14). Fluorescence spectra of the lysosomes (Fig. 2a) showed that both probes were present in the granule fraction, and resonance energy transfer from NBD to Rhodamine (21) was observed. By comparison with a calibration curve generated from phosphatidyicholine liposomes containing the fluorescent probes in different concentra-

FIG. 1. Labeling of the plasma membrane by liposomes and subsequent endocytosis. Incubation of NB4 cells with liposomes for 15 min at 4 (a) or 37 °C, followed by a 1-h chase in the absence of liposomes at 37 °C (b, c). Cells were rapidly placed between glass slides and coverslips, and microscopic observations of cells were immediately made at the wavelength of rhodamine (515–560 excitation, 590 nm long pass filter emission) using PL-Fluorat × 40 (a, b) or a PL Apo × 100 (c) oil immersion objectives on a Leica DM IRB/E fluorescence microscope.
of quenching and self-quenching of the probes at these high concentrations (Fig. 2a). The fluorescent probes were not degraded or otherwise chemically altered by their incorporation into lysosomes (Fig. 2b). Unlabeled phagosomes then were produced by nitrogen cavitation of NB4 cells that had taken up latex beads, separated from other cellular material by flotation of the latex bead-containing phagosomes on sucrose density gradients (1), and pelleted by centrifugation to remove the sucrose.

Upon mixing of labeled lysosomes with unlabeled phagosomes at 37 °C, little or no increase in fluorescence, measured as described under “Experimental Procedures,” was seen, indicating that there was no fusion, exchange of fluorescent material, or DOTAP-mediated lipid mixing between the membranes under these circumstances (Fig. 3c). However, when cytosol from neutrophils activated by phagocytosis of opsonized zymosan was added, an increase in fluorescence was observed. Dilution of the lysosomal membrane lipids into the much larger phagosomal membrane by fusion would result in the almost complete quenching of the fluorescent probes in the membrane of lysosomes and their chemical stability during pulse chase and fusion. Notice that the N-NBD-PE present in lysosomes is completely quenched by the N-Rhodamine-PE and self-quenched at these high concentrations, and therefore the lysosomes are not fluorescent when excited at the wavelength of N-NBD-PE. The somewhat lower Rhodamine peak after liberation of the probes from the lysosomes is probably due to absorption by proteins present in the sample. Panel b, thin layer chromatogram, showing the fluorescence of the probes visualized by illumination with ultraviolet light. Lane 1, N-NBD-PE standard; lane 2, lipid extract from lysosomes, labeled by the pulse-chase protocol; lane 3, from lysosomes incubated with activated cytosol for 30 min at 37 °C; lane 4 from lysosomes incubated with non-activated cytosol for 30 min at 37 °C; lane 5, N-Rhodamine-PE standard; lanes 2–4, upper spot: N-Rhodamine-PE, lower spot: N-NBD-PE. Standards contain 0.5 nmol and the other lanes, 20 nmol of phospholipids.

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FIG. 2. Dilution of the liposomal fluorescent probes in the membrane of lysosomes and their chemical stability during pulse chase and fusion. Panel a, emission spectra of liposomes (a, b) and lysosomes (c, d) before (b, c) and after (a, d) addition of the detergent Triton X-100, with excitation of N-NBD-PE at 465 nm, normalized to the NBD peak after addition of Triton. Notice that the N-NBD-PE present in liposomes is completely quenched by the N-Rhodamine-PE and self-quenched at these high concentrations, and therefore the liposomes are not fluorescent when excited at the wavelength of N-NBD-PE. The somewhat lower Rhodamine peak after liberation of the probes from the lysosomes is probably due to absorption by proteins present in the sample. Panel b, thin layer chromatogram, showing the fluorescence of the probes visualized by illumination with ultraviolet light. Lane 1, N-NBD-PE standard; lane 2, lipid extract from lysosomes, labeled by the pulse-chase protocol; lane 3, from lysosomes incubated with activated cytosol for 30 min at 37 °C, lane 4 from lysosomes incubated with non-activated cytosol for 30 min at 37 °C, lane 5, N-Rhodamine-PE standard; lanes 2–4, upper spot: N-Rhodamine-PE, lower spot: N-NBD-PE. Standards contain 0.5 nmol and the other lanes, 20 nmol of phospholipids.

FIG. 3. Fusion between labeled lysosomes and unlabeled phagosomes from human neutrophils. Panel a, 3.2 μg (protein) of a phagosome preparation were mixed with 5 μg of a lysosome preparation in buffer containing 145 mM KCl, 2.5 mM MgCl₂, 10 mM HEPES/KOH, 70 μM GTP, 200 μM ATP, and an ATP-regenerating system as described in Ref. 37 at pH 7.4, 37 °C in a total volume of 1.6 ml (curve 3). Additionally, the mixture contained 300 μg of cytosol (curve 2) or cytosol from neutrophils activated by A23187 and PMA (curve 1). Panel b, conditions as in panel a, but in the presence of cytosol from neutrophils activated by phagocytosis of opsonized zymosan (curve 1) or non-activated cytosol (curve 2). The addition of GTP and ATP or an ATP-regenerating system was not essential for fusion. Panel c, average initial rates of fluorescence increase ± 1 S.D.; the number of phagosome/lysosome preparations is indicated.
complete dequenching of the NBD, and therefore a 1% increase in fluorescence would mean that 1% of the lysosomes fused with the phagosomes. In the presence of cytosol, the initial rate of fluorescence increase was 4.2%/h (Fig. 3c). Importantly, addition of cytosol from NB4 cells activated by using a calcium ionophore and PMA tripled the activity to 12.6%/h (Fig. 3a), reaching a final level of 25% after several hours. Similar results were obtained using cytosol prepared from cells activated by phagocytosis of zymosan opsonized with human serum (Fig. 2b). After 1 h in the presence of non-activated cytosol, the fluorescence increase was 2.5%, compared with 8.2% for activated cytosol. We found that the removal of sucrose from the phagosome preparation was essential for activity. Lowering the concentration of phagosomes decreased the rate and extent of fusion, whereas increasing the concentration of lysosomes had no effect, indicating that a limited number of lysosomes fuse with phagosomes. The increase in fluorescence was not due to chemical changes in the probes caused by their incubation with cytosol (Fig. 2b).

The above experiments were performed with a rather crude preparation of lysosomes obtained by differential centrifugation. Considering that phagosome-lysosome fusion has been much harder to reconstitute in vitro than phagosome-endosome fusion (15), the observed activity could have been due to contamination of this preparation by endosomes. Endosomes were separated from the crude lysosome preparation and identified by the presence of endosomal markers as previously published (25). Endosomes were found to induce an increase in fluorescence when mixed with phagosomes; a 3.2-μg preparation of endosomes produced a 9.6%/h increase in fluorescence, compared with 12%/h for 3.2 μg of purified lysosomes. The endosomal fraction was found to represent 2.1% of the protein present in the purified lysosomal fraction. Therefore, the crude and purified lysosome preparations had similar fusion activities, and a maximum of 1.6% of the total increase in fluorescence measured with the crude lysosome preparation was contributed by contaminating endosomes.

The addition of GTP and ATP or an ATP-regenerating system to the cytosol was not essential for the fluorescence increase, in contrast to what was reported for phagosome-lysosome fusion in permeabilized J774 cells (26). However, in our system, activated cytosol was added at concentrations that produced maximal extents of fluorescence increase. In the presence of less cytosol, the initial rate of fluorescence increase was similar, but the final extent of fusion was lower, indicating that factors contained in the cytosol are present in sufficient quantities to support the activity initially, but soon become exhausted.

Intercellular membrane traffic is regulated by GTPases. These are thought to act as switches, actuated by a conforma-
ional change in the protein upon GTP hydrolysis. To test the involvement of such proteins, we used the non-hydrolysable GTP analogue GTPγS. It was found that GTPγS reduced the initial rate of fluorescence increase induced by activated cytosol to the level of non-activated cytosol (Fig. 4). Inhibition at a comparable GTPγS concentration was seen for phagosome-lysosome fusion in permeabilized J774 cells (26) while fusion of endosomes with phagosomes was shown to be activated by GTPγS (27), confirming that fusion with endosomes does not contribute significantly to the measured increase in fluorescence.

During phagocytosis-linked lysosome fusion in neutrophils and macrophages, the Src family kinase Hck translocates to phagosomes and is activated. Src family kinases are specifically inhibited by PP1 (28). To test the involvement of these proteins, phagosomes and lysosomes were pretreated with PP1, and fusion was measured in the presence of activated cytosol and PP1. It was found that PP1 inhibited the fluorescence increase with a half-maximal concentration of 1 μM (Fig. 4). Half-maximal concentrations for Src kinase inhibition in PMA stimulated intact T cells were around 26 μM (28); in human neutrophils, Hck activity was reported to be completely blocked at 10 μM (29).

Together, these data indicate that specific, efficient phagosome-lysosome fusion was reconstituted in our assay, allowing the quantitative measurement of fusion in real time. M. smegmatis inhibits the biogenesis of phagolysosomes in human neutrophils as much as pathogenic mycobacteria (11). We then sought to reconstitute the specific inhibitory effect of mycobacteria on phagosome-lysosome fusion in vitro. To this end, NB4 cells were infected with live M. smegmatis (50 bacteria per cell) by incubation for 30 min at 37 °C, followed by three washes. This incubation inhibited lysosome exocytosis as witnessed by the lack of β-glucuronidase release during the incubation, compared with β-glucuronidase release from NB4-neutrophils incubated with opsonized zymosan instead of mycobacteria (data not shown). We then prepared cytosol from these M. smegmatis-infected cells and measured fusion in the presence of mixtures of cytosol from these cells and cytosol activated as described above, compared with mixtures of activated cytosol with non-activated cytosol from uninfected cells. The lysosomes and latex bead-containing phagosomes used in these fusion experiments were prepared from non-infected cells, as in the experiments described above. Cytosol from infected cells was found to have a dose-dependent inhibitory effect on fusion (Fig. 5a), thus indicating that it contains factors inhibitory of phagosome-lysosome fusion after a brief incubation of cells with mycobacteria. Inhibition could be due to factors associated with or secreted by mycobacteria, passing into the cytosol through the membrane of the phagosomes, or the neutrophils could produce these factors after interaction with mycobacteria. To test these possibilities, cells were first incubated with heat-killed M. smegmatis, and then cytosol was prepared from these cells (Fig. 5b). No inhibition of fusion was seen. We then incubated either activated cytosol or a preparation of the plasma membranes plus the cytosol of activated cells with live M. smegmatis and used the cytosol to assay fusion. In both cases, fusion was reduced to the level of non-activated cytosol. These data suggest that factors directly involved in inhibiting fusion are present in the cytosol.

DISCUSSION

The method that we have developed for the labeling of cells with a pair of phospholipid analogues allows quantitative and kinetic measurement of phagosome-lysosome fusion in vitro, without the false positive results that are often produced by assays based on other fluorescent probes that are more easily incorporated into membranes (23). The fusion that we measured had the hallmarks of specific phagosome-lysosome fusion; it was not significantly due to contaminating endosomes and it was stimulated by cytosol prepared from cells activated by A23187 and PMA or opsonized zymosan, agents known to mobilize and induce fusion of lysosomes with the plasma membrane and phagosomes, respectively (11, 14). Phagocytosis signaling pathways are not well understood; receptor clustering, tyrosine or serine/threonine phosphorylation of substrates such as MARCKS, Ca2+ ions, and arachidonic acid are thought to be involved (30, 31). Inhibition of phagolysosome biogenesis by PP12 and other less selective tyrosine kinase inhibitors (33) suggest the involvement of tyrosine phosphorylation in intact human neutrophils. In addition we found that activation of Hck, a tyrosine kinase mostly expressed in phagocytes, correlates with phagosome-lysosome fusion in neutrophils (11, 14). In the present study, we report that PP1 reduces phagosome-lysosome fusion induced by cytosol from activated cells to the level of non-activated cytosol, providing clear evidence for the involvement of Src family tyrosine kinases in the activation of fusion.

Similar to PP1, M. smegmatis also inhibited fusion induced by activated cytosol to the level of non-activated cytosol. It seems as though there are two different types of lysosome-phagosome fusion: one that can be inhibited by these treatments and is only found in activated cells and a non-inhibitable component. However, we think that the non-inhibitable component is due to cytosol that is activated in a fraction of the cells by the act of preparing the cytosol itself, involving various manipulations of the cells, like centrifugation. We have made similar observations using neutrophils isolated from blood; a fraction of the lysosomal enzymes is secreted by non-activated cells (11).

In this paper, we provide evidence that factors associated with or released by live mycobacteria are present in the cytosol and inhibit phagosome-lysosome fusion. These factors acted when mycobacteria were simply incubated with cytosol, indicating that their phagocytosis is not required for inhibition. Once enclosed in phagosomes, these factors would have to pass the phagosomal membrane to inhibit fusion. Phagosomes containing M. bovis BCG were shown to be permeable to dextrans as large as 70 kDa from the cytoplasm in murine macrophages (34), and a number of mycobacterial surface proteins are released from phagosomes into subcellular compartments (35). Therefore, mycobacterial factors that could be inhibitors of fusion or affect fusion-regulating proteins may be released into the cytosol from phagosomes.

Additionally, the entry pathway of mycobacteria into its host cell determines the fate of the phagosome (13, 36, 32), for example by triggering specific signaling involving host cell proteins and a peripheral murine membrane protein called TACO involved in inhibition of phagolysosome biogenesis in macrophages recruited to the sites of mycobacterial entry and retained on phagosomes containing mycobacteria (9, 10). In this study, we provide evidence that inhibition of phagosome-lysosome fusion in human neutrophils also involves mycobacterial factors acting in the cytosol.

In conclusion, the in vitro reconstitution of fusion and a quantitative and kinetic assay have allowed us to identify the role of Src family tyrosine kinases in phagosome-lysosome fusion stimulated by cytosol from activated cells. Moreover, the in vitro assay could facilitate the search for the mycobacterial factors involved in inhibition of fusion and help to unravel the mechanism of fusion.

2. C. Cougoule and I. Maridonneau-Parini, unpublished data.
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