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Fusion of Azurophil Granules with Phagosomes and Activation of the Tyrosine Kinase Hck Are Specifically Inhibited During Phagocytosis of Mycobacteria by Human Neutrophils

Elsa-Noah N’Diaye,* Xavier Darzacq,* Catherine Astarie-Dequeker,* Mamadou Daffé,* Jero Calafat,† and Isabelle Maridonneau-Parini‡

Pathogenic mycobacteria paralyze macrophages and reside within phagosomes, which do not fuse with lysosomal granules. Mycobacteria are also internalized by neutrophils, which possess at least two types of granules, specific and azurophil granules, the latter being specialized lysosomes. Here, we investigated the ability of mycobacteria to inhibit the fusion of these granules with their phagosomes in human neutrophils. It was found that when pathogenic (Mycobacterium kansasii and Mycobacterium avium) or nonpathogenic (Mycobacterium smegmatis and Mycobacterium phlei) mycobacteria were internalized by neutrophils, they inhibited the induction of azurophil granule fusion with phagosomes even when they were serum opsonized. In contrast, secretion of specific granule content and production of O$_2^-$, both of which contribute to the neutrophil bactericidal response, were triggered. Hck is a Src family tyrosine kinase associated with azurophil granules. During internalization of zymosan, azurophil granules fused with phagosomes and Hck was activated and translocated to the phagosomal membrane, whereas in neutrophils engulfing mycobacteria, Hck did not translocate and remained unactivated. The activation of the tyrosine kinase Fgr was not affected. These results indicate that 1) pathogenic and nonpathogenic mycobacteria trigger similar bactericidal responses in neutrophils, 2) phagocytosis and fusion of azurophil granules can be uncoupled by mycobacteria, and 3) Hck could be one of the key elements of the azurophil secretory pathway that are altered during phagocytosis of mycobacteria. The Journal of Immunology, 1998, 161: 4983–4991.

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eutrophils and macrophages participate in host defense by three mechanisms that lead to the destruction of invading microorganisms: phagocytosis of the infectious particles, generation of toxic oxygen derivatives through the activation of the NADPH oxidase, and release of microbicidal molecules from their granules (1).

Pathogenic mycobacteria are facultative intracellular parasites that are able to survive and replicate within macrophages (2). In addition to the well-known pathogenic species Mycobacterium tuberculosis and Mycobacterium leprae, opportunistic pathogenic mycobacteria have attracted epidemiologic interest since they cause infections in immunocompromised people. Two major opportunistic species, Mycobacterium avium and Mycobacterium kansasii, have frequently been isolated from pulmonary and disseminated infections in AIDS patients (3, 4). In macrophages, Mycobacterium microti, Mycobacterium marinum, M. avium, and M. tuberculosis inhibit the fusion between lysosomal granules and their phagosomes (5–10), leading to the proposal that these properties favor their survival in macrophages (2).

Macrophages have long been regarded as the key phagocytic cells in mycobacterial infections. However, neutrophils have emerged as playing a significant protective role in tuberculosis (11–13). In contrast to macrophages, which contain only lysosomes (14), neutrophils contain at least two types of secretory granules, the azurophil and specific granules (15). Azurophil granules are a special class of lysosomes that, in addition to typical lysosomal enzymes, store bactericidal proteins and neutral proteases. Specific granules also contain proteins implicated in the bactericidal function of neutrophils, and they are a reservoir of plasma membrane receptors and cytochrome b$_5_5_8$, a component of the NADPH oxidase complex (15). Proteins controlling the process of mobilization/fusion of these granules with the phagosomal or the plasma membrane have not yet been identified. Several reports suggest that a tyrosine kinase of the Src family, Hck, the expression of which is restricted to phagocytes (16), could be involved in this process: 1) it is mainly associated with the membrane of azurophil granules (17); 2) in the course of phagocytosis of opsonized zymosan, azurophil granules fuse with phagosomes and Hck translocates to the phagosomal membrane (17); 3) during this process, Hck is activated in the granular fraction and, to a lesser extent, in the phagosomal fraction (18); and 4) tyrosine kinase inhibitors inhibit the degradation process (16).

Although neutrophils play a defensive role during mycobacterial infections (11–13), few data regarding bactericidal responses of neutrophils toward mycobacteria (19–22) are available, e.g., mobilization of granules in response to mycobacteria has not been studied. In this paper, the release of azurophil and specific granule content was determined in human neutrophils during the phagocytosis of pathogenic (M. avium and M. kansasii) and nonpathogenic (Mycobacterium phlei and Mycobacterium smegmatis) mycobacteria. The tyrosine kinase activity of Hck was also studied.

Materials and Methods

Reagents

Ficol and PMSF were purchased from Eurobio (Les Ullis, France). MEM and HEPES were purchased from Life Technologies (Cergy Pontoise, France). Leupeptin, aprotinin, pepstatin, diisopropylfluorophosphate, and FITC were purchased from Sigma (St. Louis, MO). Anti-myeloperoxidase

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Abs (23) were from CLB (Amsterdam, The Netherlands); anti-lactoferrin was from Cappel Laboratories (Cocranville, PA); and anti-rabbit Abs gold conjugates were from Amersham (s'Hertogenbosch, The Netherlands).

**Human neutrophils**

Neutrophils were isolated from the blood of healthy donors, separated by the Dextran-Ficoll method as previously described (24), resuspended in MEM, 10 mM HEPES, pH 7.4, and maintained for 20 min at 37°C before stimulation. The final suspensions contained ~95% neutrophils.

**Bacteria strains and growth conditions**

*M. phlei* (ATCC 11758), *M. smegmatis* (ATCC 607), and *M. kansasii* (ATCC 124478) (all from American Type Culture Collection (ATCC), Manassas, VA) were grown at 37°C as surface pellicles in 250-ml flasks containing 100 ml of Sauton broth medium. The culture was centrifuged at 10,000 × g for 10 min, and the pellet was resuspended in PBS. To remove clumps, the bacterial suspensions were sedimented for 10 min, and the supernatants were collected and centrifuged for 10 min at 200 × g. Supernatants were collected, counted under a microscope in a Thoma chamber, supplemented with 5% glycerol, and stored at −80°C until use. At least 90% of the mycobacteria were individualized, and the remaining 10% formed small aggregates containing two or three bacilli. *Bacillus subtilis* (ATCC 6633) was grown overnight in Tryptase-Soja broth (Biomerieux, Lyon, France) at 37°C, washed in PBS, and stored under the same conditions as the mycobacteria. All experiments were performed using a bacteria:phagocyte ratio of 50:1.

**FITC staining of bacteria**

Bacteria were stained with FITC as previously described (25). Briefly, 10⁸ bacteria were added to 1 ml 0.01% FITC in 0.2 M Na₂CO₃/NaHCO₃ buffer, pH 10.2, for 10 min. The bacteria were then washed twice in PBS, pH 7.4.

**Opsonization of bacteria and zymosan**

Bacteria or zymosan were incubated in pooled human sera for 20 min at 37°C, washed twice with PBS, pH 7.4, and resuspended in the same buffer containing 1 mM CaCl₂ and 0.5 mM MgCl₂ (24). Opsonization was checked by incubating bacteria or zymosan with FITC-coupled rabbit Abs directed against human IgG. Fluorescence was measured by FACScan analysis (Becton Dickinson, San Jose, CA).

**Phagocytosis measurement and indirect immunofluorescence**

Neutrophils (7 × 10⁶/ml) adhering on glass coverslips were incubated at 37°C for 20 min in MEM-HEPES as previously described (26) and exposed to FITC-stained mycobacteria. Cells were then washed and fixed with 3.7% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 30 min at room temperature. After neutralizing with 50 mM NH₄ Cl, with 3.7% paraformaldehyde in PBS containing 15 mM sucrose, pH 7.4, posed to FITC-stained mycobacteria. Cells were then washed and fixed at 37°C for 20 min in MEM-HEPES as previously described (26) and exocytosed. The final suspensions contained ~95% neutrophils.

**Immuno-electron microscopy**

Neutrophils were fixed in 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h and then pelleted in 10% (w/v) gelatin in PBS. Ultrathin frozen sections were incubated at room temperature with mouse monoclonal anti-human myeloperoxidase (1:200) (23) or rabbit anti-human lactoferrin (1:400) followed by incubation with secondary Abs linked to 10-nm gold particles (dilution 1:40). All incubations were performed for 1 h. Ultrathin cryosections incubated with irrelevant control rabbit or murine Abs under the same conditions produced negligible background labeling. After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope (Eindhoven, The Netherlands).

O₂⁻ production

The production of O₂⁻ was measured using the superoxide dismutase-inhibitable ferricytochrome c reduction method, as previously described (28).

**Immunoblotst**

Control or stimulated neutrophils (5 × 10⁶/ml) were pelleted and the supernatants were centrifuged (10,000 × g for 10 min) to eliminate bacteria. Cell pellets lysed in Triton X100 and cell supernatants were stored at −80°C until used. Lysozyme was measured in the extracellular medium and in the cells by the following the hydrolysis of Microcococcus sp at 450 nm, as previously described (29). Myeloperoxidase and lactoferrin were measured by ELISA as previously described (17). The enzyme activity of β-glucuronidase was measured at 405 nm as previously described (30).

In some experiments, for quantification of cell death, release of the cytosolic enzyme lactate dehydrogenase was measured using the colorimetric assay kit from Boehringer (Meylan, France) according to the manufacturer’s instructions.

**Results**

**Internalization of mycobacteria by human neutrophils**

The ability of neutrophils to engulf pathogenic and nonpathogenic mycobacteria was examined. Human neutrophils internalized both pathogenic and nonpathogenic mycobacteria under nonopsonic conditions (Fig. 1). Opsonization of mycobacteria in human serum enhanced the percentage of cells with engulfed bacteria (Fig. 1).

**Selective inhibition of azurophil granule exocytosis by mycobacteria**

We first examined whether neutrophils degranulate when exposed to mycobacteria. The release of lysozyme, a hydrolyase present predominantly in specific but also in azurophil granules (1), was measured. In these experiments and the following, opsonized zymosan was used as a positive control. Pathogenic as well as nonpathogenic mycobacteria induced the exocytosis of lysozyme to similar degrees (Fig. 2). Unopsonized mycobacteria (Fig. 2) and rabbit anti-human lactoferrin (1:400) followed by incubation with secondary Abs linked to 10-nm gold particles (dilution 1:40). All incubations were performed for 1 h. Ultrathin cryosections incubated with irrelevant control rabbit or murine Abs under the same conditions produced negligible background labeling. After immunolabeling, the cryosections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope (Eindhoven, The Netherlands).

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In some experiments, for quantification of cell death, release of the cytosolic enzyme lactate dehydrogenase was measured using the colorimetric assay kit from Boehringer (Meylan, France) according to the manufacturer’s instructions.

**Kinase assays**

Proteins were solubilized from neutrophils with a buffer containing 2% Nonidet P-40 and cytosol from NB4 cells to avoid proteolysis, as previously described (17). Kinases were immunoprecipitated and assayed for in vitro kinase activity in the presence of acid-treated enolase as exogenous substrate, 10 mM MnCl₂, 10 μM MgCl₂, and 10 μCi of [γ-³²P]ATP (6000 mCi/mmol) as described (17, 31). The specificity of the rabbit anti-Hck antisem has been characterized previously (17), and affinity-purified anti-Fgr and anti-Lyn IgG (Santa Cruz Biotechnology) were used as described previously (31). The radioactivity incorporated in enolase was quantified using the ImageQuant program on the Storm840 imager, Molecular Dynamics (Sunnyvale, CA).

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To examine the mobilization of azurophil and specific granules independently, exocytosis of myeloperoxidase, an azurophil granule marker, and lactoferrin, a specific granule marker (15), were
measured. As observed in Figure 3, while the release of lactoferrin was stimulated in response to pathogenic or nonpathogenic mycobacteria, the exocytosis of myeloperoxidase was insignificant except for opsonized *M. smegmatis* (Fig. 3), the effect of which was, however, negligible. This could not be attributed to a defective capacity of neutrophils to release myeloperoxidase, as attested to by the cell response to opsonized zymosan. The possibility that serum opsonization of mycobacteria could elicit the release of myeloperoxidase was tested. As shown in Figure 3, neither myeloperoxidase nor lactoferrin secretion was enhanced in response to opsonized mycobacteria. To test the possibility that this phenomenon was restricted to mycobacteria, *B. subtilis*, another Gram-positive bacterium, was used in parallel. We observed that *B. subtilis* was ingested by neutrophils with an efficiency similar to mycobacteria (data not shown), but in contrast, it triggered the release of both myeloperoxidase and lactoferrin (Fig. 4). Opsonized or nonopsonized *B. subtilis* induced similar responses (Fig. 4). The low level of release of azurophil granule content in the presence of mycobacteria was confirmed using another marker: the lysosomal enzyme $\beta$-glucuronidase (15). Like myeloperoxidase, $\beta$-glucuronidase was efficiently released by neutrophils in response

**FIGURE 1.** Phagocytosis of mycobacteria by human neutrophils. FITC-labeled mycobacteria were serum-opsonized (*ops +* ) or not opsonized (*ops −* ). Neutrophils were exposed to mycobacteria (cell:bacteria ratio, 1:50) for 40 min at 37°C. Cells were washed and fixed, and nonphagocytosed mycobacteria were quenched using trypan blue as described in Materials and Methods. The percentage of cells with ≥1 fluorescent bacterium was determined by fluorescence microscopy (see Fig. 8). *M. smeg.*, *M. smegmatis*. Results are expressed as mean ± SD of three independent experiments.

**FIGURE 2.** Pathogenic and nonpathogenic mycobacteria trigger the release of lysozyme. Resting neutrophils (C, control) and neutrophils exposed to serum-opsonized (*ops +* ) or nonopsonized (*ops −* ) mycobacteria (cell:bacteria ratio, 1:50) or to 3 mg/ml opsonized zymosan (Z), as a positive control, were incubated for 40 min at 37°C. The release of lysozyme is expressed as the percent of the total lysozyme cell content (mean ± SD of three experiments). *, $p < 0.05$ when compared with control calculated with unpaired Student’s *t* test.

**FIGURE 3.** Pathogenic and nonpathogenic mycobacteria trigger the release of lactoferrin but not myeloperoxidase. Neutrophils were stimulated for 40 min as described in the legend to Figure 2. The release of lactoferrin, a specific granule marker, and myeloperoxidase, an azurophil granule marker, was measured by ELISA. Results are expressed as mean ± SD of at least four experiments. *, $p < 0.05$ when compared with control calculated with unpaired Student’s *t* test.
to zymosan or B. subtilis, but only a limited and insignificant release was observed in response to pathogenic and nonpathogenic mycobacteria (Fig. 5). During the course of these experiments, cell viability was measured by the release of the cytosolic enzyme lactate dehydrogenase. In control cells or cells exposed to zymosan, the release was 4% and in neutrophils exposed to mycobacteria, 6 to 8%. It is possible that the low level of release of azurophil markers from mycobacteria-infected neutrophils results from the higher percentage of dead neutrophils.

The azurophil granule marker myeloperoxidase is not delivered to phagosomes containing mycobacteria

Secretion of granule contents to the external milieu may occur subsequent to granule fusion with unsealed phagosomes. To determine whether the absence or low release of azurophil granule markers in the presence of mycobacteria correlated with a limited fusion with phagosomes, immuno-electron microscopy was performed on neutrophils infected by M. smegmatis and M. kansasii, compared with B. subtilis and zymosan. Figure 6 shows that myeloperoxidase and lactoferrin were present in phagosomes containing zymosan or B. subtilis. In contrast, phagosomes containing mycobacteria were very poorly stained by anti-myeloperoxidase Abs (Fig. 6), whereas they were strongly lactoferrin positive. This finding demonstrates that phagosomes containing mycobacteria are permissive for specific granule fusion but do not fuse with azurophil granules.

Neutrophils infected by mycobacteria generate O$_2^-$

The possibility that mycobacteria could alter another function contributing to the bactericidal response of neutrophils was investigated. The production of O$_2^-$ was therefore examined in cells infected by mycobacteria or exposed to zymosan. O$_2^-$ generation, continuously measured in response to mycobacteria (Fig. 7A), increased linearly until a plateau was reached between 90 and 120 min depending on the blood donor. Next, experiments were performed at 40 min to measure the production of O$_2^-$ at the initial rate. When neutrophils were exposed to pathogenic or nonpathogenic mycobacteria, the O$_2^-$ generation was elicited to a similar magnitude, and again, serum opsonization of mycobacteria did not affect the results (Fig. 7B). We verified the functional assembly of the O$_2^-$-producing enzyme NADPH oxidase, which requires translocation of cytosolic components to the plasma membrane (32).

The Src tyrosine kinase Hck does not translocate to the phagosomal membrane and is not activated in mycobacteria-infected neutrophils

The Src family tyrosine kinase Hck is associated with the membrane of azurophil granules, is activated during the process of exocytosis, and translocates to the phagosomal membrane (16–18). Therefore, we investigated whether the translocation of Hck toward phagosomes and its activation occurred during the phagocytosis of mycobacteria. When indirect immunofluorescence was performed, no Hck staining was detectable at the phagosomal membrane around FITC-stained mycobacteria (Fig. 8, middle panels), while Hck was clearly present at the phagosomal membrane in neutrophils that had internalized zymosan (Fig. 8, upper panel). The possibility that Hck could be present at the phagosomal membrane but hidden by the fluorescence of mycobacteria was ruled out, because similar results were obtained with nonfluorescent M. phlei (data not shown) or when Hck was stained with rhodamine-coupled Abs and mycobacteria stained with FITC (Fig. 8, lower panels). When mycobacteria were serum opsonized, Hck did not translocate to the phagosomal membrane either (data not shown).

The kinase activity of Hck was measured along the process of phagocytosis using either zymosan, M. phlei, or M. kansasii at the same cell:particle ratio. In neutrophils stimulated by zymosan, the kinase was activated with magnitude, and kinetics similar to those observed previously in human neutrophils exposed to opsonized zymosan (18). However, Hck kinase activity remained practically unchanged in response to M. phlei and M. kansasii, except after 20 min of exposure to M. phlei, which induced a significant but negligible increase in the kinase activity (Fig. 9). Again, there was no difference between opsonized and nonopsonized M. phlei (results

![FIGURE 4.](http://www.jimmunol.org/) B. subtilis stimulates exocytosis of both specific and azurophil granule markers. Neutrophils were stimulated or not (control) with serum-opsionized (ops) and nonopsionized (ops-) B. subtilis (cell:bacteria ratio, 1:50) for 40 min, and the release of lactoferrin and myeloperoxidase was measured by ELISA. Results are expressed as mean ± SD of five experiments. * p < 0.05 when compared with control calculated with unpaired Student’s t test.

![FIGURE 5.](http://www.jimmunol.org/) Mycobacteria do not trigger the release of β-glucuronidase in neutrophils. Neutrophils were stimulated for 40 min with mycobacteria, B. subtilis (cell:bacteria ratio, 1:50) or 3 mg/ml opsonized zymosan (OZ). The release of β-glucuronidase is expressed as the percent of the total β-glucuronidase cell content (mean ± SD of three or four experiments). * p < 0.05 when compared with control calculated with unpaired Student’s t test.
FIGURE 6. Lactoferrin, but not myeloperoxidase, is released in mycobacteria-containing phagosomes. Neutrophils were exposed to opsonized zymosan (cell:particle ratio, 1:10), B. subtilis or mycobacteria for 30 min at 37°C (cell:particle ratio, 1:50), washed, and further incubated in particle-free medium for 15 min before fixation. Zymosan, Cryosections of neutrophils infected with opsonized zymosan. Upper panel, Lactoferrin staining (Lf), and lower panel, myeloperoxidase staining (MPO). Phagosomes (ph) are labeled with both markers. Insets, Higher magnification of areas indicated by asterisks. No background labeling is observed in the nucleus (n). Bars = 400 nm; insets = 200 nm. Bacillus, Neutrophils infected with Bacillus subtilis (same as zymosan). Mycobacteria, Neutrophils infected with M. smegmatis (upper panel and lower panel, right) or with M. kansasii (lower panel, left). Phagosomes (ph) containing mycobacteria were highly lactoferrin positive and essentially myeloperoxidase negative; granules (g) are positive for myeloperoxidase. Bar = 400 nm.
To determine whether this inhibition was Hck specific, the kinase activity of another Src family tyrosine kinase, Fgr, the expression of which is also restricted to phagocytes (16), was measured. We have previously reported that Fgr is activated during phagocytosis of opsonized zymosan (31). Here, we observed that, during phagocytosis of nonopsonized zymosan, \( M. \) \( \textit{phlei} \), \( M. \) \( \textit{kansasii} \), or \( M. \) \( \textit{phlei} \), \( M. \) \( \textit{kansasii} \), or \( M. \) \( \textit{phlei} \), Fgr was activated (Fig. 9), indicating that not all of the members of the Src family are affected during phagocytosis of mycobacteria. This conclusion was further supported by the observation that Lyn was activated in neutrophils exposed to zymosan or \( M. \) \( \textit{phlei} \) (data not shown). Taken together, these results show that, during their phagocytosis, mycobacteria interfere with translocation of Hck to the phagosomes and its activation.

**Discussion**

Although neutrophils play a defensive role during mycobacterial infections (11–13), very few data regarding bactericidal responses of neutrophils toward mycobacteria are available (19–22), and no comparative studies between pathogenic and nonpathogenic bacteria have been performed. We show here that both pathogenic and nonpathogenic mycobacteria are internalized by neutrophils. Opsonization enhanced phagocytosis of opsonized zymosan (31). Here, we observed that, during phagocytosis of nonopsonized zymosan, \( M. \) \( \textit{phlei} \), \( M. \) \( \textit{kansasii} \), or \( M. \) \( \textit{phlei} \), Fgr was activated (Fig. 9), indicating that not all of the members of the Src family are affected during phagocytosis of mycobacteria. This conclusion was further supported by the observation that Lyn was activated in neutrophils exposed to zymosan or \( M. \) \( \textit{phlei} \) (data not shown). Taken together, these results show that, during their phagocytosis, mycobacteria interfere with translocation of Hck to the phagosomes and its activation.

**Figure 7.** Production of \( \text{O}_2^- \) and assembly of NADPH oxidase components in response to mycobacteria. *A.* Production of \( \text{O}_2^- \) in response to zymosan or mycobacteria (50 particles/cell) was continuously measured by the superoxide dismutase-inhibitable reduction of ferricytochrome \( c \). One representative experiment is shown. *B.* Neutrophils were incubated for 40 min with serum-opsonized (ops +) and nonopsonized (ops −) mycobacteria. In each experiment, cells were stimulated with 3 mg/ml opsonized zymosan (Z) for 20 min; C indicates resting neutrophils. Results are expressed as the mean ± SD of three to five experiments. *, \( p < 0.05 \) when compared with control calculated with unpaired Student’s *t* test. *C.* NADPH oxidase cytosolic components, p47phox and p67phox, translocate to the plasma membrane of mycobacteria-exposed neutrophils. Resting neutrophils (C for control) or neutrophils stimulated with 3 mg/ml opsonized zymosan for 20 min or with \( M. \) \( \textit{smegmatis} \) for 40 min were disrupted and fractionated by differential centrifugation. Proteins from the membrane (m) and cytosol (c) were immunoblotted using rabbit antisera directed against p47phox or p67phox. One representative experiment is shown.
was released, whereas azurophil granules were very poorly or not at all mobilized. When *B. subtilis* and mycobacteria were used at the same bacteria:cell ratio, they were internalized with similar efficiency, but exocytosis of azurophil granule markers was detected only in response to *B. subtilis*. Immuno-electron microscopy confirmed the lack of azurophil granule fusion, showing that phagosomes containing either pathogenic or nonpathogenic mycobacteria were essentially myeloperoxidase negative, whereas phagosomes containing zymosan or *B. subtilis* were myeloperoxidase positive. In macrophages, inhibition of the fusion between lysosomes and phagosomes has been essentially studied with pathogenic species (*M. microti*, *M. marinum*, *M. avium*, and *M. tuberculosis*) (5–10). A report on the nonpathogenic species *Mycobacterium aurum* shows that lysosome-phagosome fusions are comparable with that of *M. avium* after a 24-h infection (7). In the light of our results on neutrophils, it would be important to further investigate the release of lysosomal markers in macrophages infected with nonpathogenic mycobacteria to establish whether this inhibition is restricted to pathogenic species. This knowledge would be helpful in the understanding of mycobacterial pathogenicity.

Attempts have been made to identify membrane-associated proteins that could differentially regulate the mobilization and fusion of specific and azurophil granules. We and others have shown that several proteins, due to their unique localization on azurophil or specific granule membranes, may be implicated in these regulations (14, 15, 17, 24, 26). Until now, only two proteins that could play a role in exocytosis have been identified on the surface of azurophil granules: leukophysin, a protein related to synaptophysin...
(39), and the tyrosine kinase Hck (17). The exocytosis of azurophil granules is inhibited by tyrosine kinase inhibitors (16). Moreover, Hck, which is mainly expressed in phagocytes, is associated with the membrane of azurophil granules, translocates to the phagosome membrane during internalization of either opsonized zymosan (17, 18) or zymosan (this report), and is concomitantly activated (18). When neutrophils are activated by FMLP or PMA, which induce activation of NADPH oxidase and the release of specific but not of azurophil granules, Hck is not activated (18). In contrast to soluble stimuli, which do not or only poorly mobilize azurophil granules, phagocytosis of particles is accompanied by secretion of this granule type. Here we show that when mycobacteria are phagocytosed by neutrophils, there is almost no release of azurophil granule markers in the extracellular space or inside phagosomes. Under these conditions, Hck does not translocate to the phagosomal membrane, and its level of tyrosine kinase activity remains very low. Taken together, these results support the hypothesis that Hck might play a critical role in the mobilization of azurophil granules toward phagosomes. In HL60 cells differentiated in macrophages and in human monocytes, Hck is also present on cytoplasmic granules, probably lysosomes (17). It is possible, therefore, that Hck is also implicated in the regulation of lysosome mobilization in these cells. Protein phosphorylation on tyrosine residues during phagolysosome biogenesis has been observed on phagosomes isolated from murine macrophages (40). Interestingly, we have previously shown that during the mobilization of azurophil granules in response to phagocytosis of opsonized zymosan, Hck is activated in the azurophil granule fraction but also in the fraction enriched in phagosomes. Therefore, these data strongly suggest that Hck could phosphorylate substrates on phagosomes and/or on azurophil granules. Identification of these proteins will help the understanding of the function of Hck.

To explain why Hck is not activated when neutrophils internalize mycobacteria, one could postulate that membrane receptors involved in their phagocytosis do not evoke signalization for Hck activation. However, this seems unlikely because zymosan and opsonized zymosan use distinct receptors, possibly the lectin site of CR3 (41) and a putative β-glucan receptor (42) for the former and complement and IgG receptors (43) for the latter; but in both cases, similar Hck activation is observed (see Ref. 18 and this report). Moreover, opsonized or nonopsonized mycobacteria use distinct receptors (2, 44) but do not trigger Hck activation, suggesting that the control of Hck tyrosine kinase activity during phagocytosis might be independent of the receptor used. Alternatively, mycobacteria could either interfere with downstream receptor degradation signals or directly inhibit Hck. Recently, a factor located in the periplasmic compartment of Escherichia coli and secreted in the culture medium, which is inhibitory for the Sre family tyrosine kinase p56<sup>lck</sup>, has been identified (45). A similar activity, with a tropism for Hck, given that Fgr and Lyn were not affected, could also exist in mycobacteria. This possibility is currently under investigation in our laboratory.

In conclusion, our results show that when human neutrophils are exposed to mycobacteria, they exhibit the typical early bactericidal responses: phagocytosis, generation of O<sub>2</sub><sup>-</sup>, and exocytosis of specific granules. In contrast, mobilization of azurophil granules is not triggered by mycobacteria even when they are opsonized. This is an important finding as it shows that phagocytosis and fusion of azurophil granule with phagosomes can be dissociated events that are uncoupled by mycobacteria. In addition, we demonstrate that neutrophils exhibit similar bactericidal responses to opsonized or nonopsonized, pathogenic and nonpathogenic mycobacteria. Finally, we propose that Hck could be one of the key elements of the secretory pathway that are altered during phagocytosis of mycobacteria.

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References


