Membrane retrieval in neutrophils during phagocytosis: inhibition by M protein-expressing S. pyogenes bacteria

Susanne Bauer and Hans Tapper

Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, BMC, B14, Lund University, Sweden

Abstract: During phagocytosis and phagosome maturation, complex membrane traffic events must be coordinated. We have observed, using fluorescent fluid-phase and membrane markers, that in the human neutrophil, internalization of nonopsonized, Gram-positive bacteria, but not of latex beads, is accompanied by a rapid and localized formation of pinosomal structures. This pinocytic response is calcium-dependent but insensitive to actin cytoskeleton disruption and wortmannin treatment. Contrary to what we observe, endosomal structures usually are considered to participate in phagosome formation by providing necessary membrane to form phagosomes. Instead, our results show a coupling between neutrophil secretory and membrane-retrieval processes during phagosome maturation, and we suggest that the observed, localized pinocytic response is linked to the secretion of azurophilic granules toward nascent phagosomes. Accordingly, M and M-like protein-expressing Streptococcus pyogenes bacteria, which are able to survive inside neutrophil phagosomes, inhibit the secretion of azurophilic granules to phagosomes and pinosome formation. J. Leukoc. Biol. 76: 000–000; 2004.

Key Words: membrane traffic · pinocytosis · secretion · localization · azurophilic granules

INTRODUCTION

Professional phagocytes, e.g., neutrophils and macrophages, express an array of specialized phagocytic receptors that recognize conserved patterns or opsonins on the surface of microorganisms. These receptors are important for our innate immune defense against invading pathogens and can trigger phagocytosis as well as other antimicrobial responses [1]. The killing of internalized bacteria is accomplished by a number of complementary mechanisms, such as the oxidative burst and the fusion of various endocytic organelles with phagosomes, which progressively are acidified and mature into bactericidal phagolysosomes [2–6].

The formation of a phagosome requires that membrane is provided at the site of particle attachment by localized secretion of various granule types [7], endosomal compartments [8], or endoplasmic reticulum [9]. The phagosome then matures, after its scission from the plasma membrane, through a sequence of fusion and fission reactions, which in many respects, resembles the progression of the endocytic pathway [10]. This phagosomal maturation process requires kinase activation, alterations in phospholipid metabolism, remodeling of the actin cytoskeleton, and acceleration of membrane traffic, processes that trigger and guide the fusion of elements of the endocytic pathway. Regulatory signals target cytoskeletal elements and various docking and fusion/fission components, whereas various Rab proteins and phosphoinositide metabolites determine the specificity and localization of the fusion events. During the interactions of the nascent vacuole with the endocytic pathway, many phagosomal content proteins and phagosomal transmembrane proteins are progressively acquired, whereas others are removed. Notably, the phagosomal delivery of granule contents and granule membrane components must be balanced with a continuous removal of membrane and fluid to maintain the size of phagosomes. One mechanism by which this could occur was suggested by the “kiss-and-run” hypothesis, according to which transient and incomplete fusion events allow intermingling of granules and phagosomes [11]. Recently, the molecular complexity of phagosomes has been demonstrated by the use of powerful approaches, such as proteomic analysis and phagosome antibody libraries [12, 13]. Endogenous ligands on nonopsonized targets or opsonins can trigger different signaling pathways, and immunoglobulin G (IgG)-mediated and complement-mediated phagocytosis have been shown to differ mechanistically [14, 15]. Furthermore, adding to the complexity, many pathogens are able to modulate the phagosomal maturation sequence. This can occur through an inhibition of phagosome-lysosome fusion as has been described for a number of pathogenic bacteria such as Mycobacterium tuberculosis [16, 17], Legionella pneumophilia [18], Brucella abortus [19], and Bordetella pertussis [20].

Neutrophils are considered to be our first line of defense against invading pathogens as a result of their rapid mobilization and response toward bacterial peptides and inflammatory cytokines [21, 22]. For neutrophils to generate a bactericidal
phagosome, several types of specialized secretory organelles are important, in particular, the primary (azurophilic) and secondary (specific) granules [23]. Phagocytosis of IgG-opsonized zymosan particles or latex beads was recently demonstrated to induce a rapid pinocytic response in human neutrophils. This burst of fluid-phase pinocytosis, triggered by Fc receptor for IgG (FcγR)-mediated phagocytosis, was shown to be spatially restricted to the vicinity of the forming phagosome. Furthermore, the pinocytosis process seemed to precede and to be independent of phagosomal sealing [24]. It was speculated that the pinosome formation was a consequence of excess delivery of granule membrane and merely a compensatory mechanism to maintain the size of the formed phagosome. A polarized sequestration of plasma membrane in intracellular vesicles during the early stages of IgG-mediated phagocytosis has been demonstrated also in monocytes, where these vesicles were suggested to provide membrane for the forming phagosome [25].

The uptake of particulate matter by phagocytosis occurs mainly in professional phagocytic cells [3]. However, the endocytic uptake of soluble substances, by fluid-phase or by receptor-mediated pinocytosis, can be performed more or less efficiently by all cells [26]. Pinosomes are small vesicles derived from the plasma membrane, primarily by COPI-, clathrin-, and caveolae-dependent mechanisms [27]. The pinosomal compartment is progressively acidified, and internalized material rapidly enters sorting endosomes, acidic pH of approximately 6, by mechanisms dependent on Rab5 and early endosome antigen 1 (EEA1). Recently, adenosine 5′-diphosphate ribosylation factor 6-dependent delivery of endosomal vesicles to sites of phagocytosis has been described [28].

*Streptococcus pyogenes* is a gram-positive human pathogen responsible for a wide spectrum of diseases ranging from uncomplicated pharyngitis and pyoderma [29–31] to severe and life-threatening, invasive diseases such as sepsis and streptococcal toxic shock syndrome [32–36]. *S. pyogenes* bacteria can survive an incubation in human blood [37], and this has in part been ascribed to an antiphagocytic effect of M and M-like proteins, was generated from the wild-type serotype as described previously [42]. From a culture of *S. pyogenes* on blood Todd-Hewitt agar (API) or Todd-Hewitt agar (BM71), a few colonies were transferred to Todd-Hewitt broth at 37°C in 5% CO₂. For BM71, 5 µg ml⁻¹ tetracycline was added to the culture medium. After an overnight growth at 37°C, the bacteria were harvested and washed three times in phosphate-buffered saline (PBS) containing 116 mM NaCl, 1.7 mM KH₂PO₄, and 4.9 mM Na₂HPO₄. Bacterial suspensions were adjusted (photometrically optical density of 620) to the desired concentration. The viability of the bacteria (>95% viability) was verified using a live-dead kit from Molecular Probes. For some experiments, bacteria were killed by heating in a water bath at 80°C for 10 min.

**Neutrophil isolation**

Human polymorphonuclear leukocytes were freshly prepared, as described previously [40], from heparinized human blood, kindly provided by healthy volunteers. Briefly, whole blood was layered on Polymorphprep™ and centrifuged at 400 g for 35 min at 18°C. The neutrophil layer was recovered and suspended in 50 ml calcium- and magnesium-containing PBS. After centrifugation at 350 g for 10 min, contaminating erythrocytes were removed by hypotonic lysis for 20 s. The cells were then pelleted at 250 g (5 min), counted using a hemocytometer, and resuspended in MEM at a concentration of 10⁷ cells ml⁻¹. After purification, neutrophils were gently rotated end-over-end at room temperature. All experiments were performed in Na medium (containing 5.6 mM glucose, 127 mM NaCl, 10.6 mM KCl, 2.4 mM KH₂PO₄, 1.6 mM MgSO₄, 10 mM Hepes, and 1.8 mM CaCl₂; the pH was adjusted to 7.3 with NaOH) within 2 h of isolation.

**Phagocytosis/pinocytosis assay**

To synchronize interaction, bacteria and neutrophils in Na medium containing 1 mM Ca²⁺ were pelleted in a microcentrifuge at 12,000 g for 20 s and were then incubated at 37°C for 40 s. The cell/bacteria pellet was resuspended in new medium, and the cells were kept at 37°C for various times to allow the phagocytic process. Thereafter, incubation was continued at 37°C in the presence of Lucifer Yellow (1 mg/ml) for 1 min or FM¹-4-36FX (10 µM) for 30 s. After experimentation, samples were washed twice with ice-cold PBS, fixed, and further processed.

**Materials and methods**

**Reagents**

Lucifer Yellow, FM¹-4-36FX, and ALEXA Fluor® 594 (Fab)2 fragment of goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Zymosan, cytochalasin D, ionomycin, piperazine bis-2-ethane sulfonic acid, poly-L-lysine, and goat serum were obtained from Sigma-Aldrich (St. Louis, MO). Polyethylene latex beads (2 µm) were obtained from Polysciences (Warrington, PA). Dako-mounting medium was purchased from Dako (Carpinteria, CA). Rabbit anti-human lysosome-associated membrane protein (LAMP)-3 (CD63) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antistreptococcal antiserum was from a rabbit immunized with whole *S. pyogenes* bacteria. Minimum essential medium (MEM) was obtained from Life Technologies (Taby, Sweden). Polymorphprep™ was from Axis-Shield PoC AS (Oslo, Norway).

**Bacterial strains**

The wild-type *S. pyogenes* activated protein 1 (API; 40/58) strain of the M₁ serotype was provided by the World Health Organization Streptococcal Reference Laboratory (Prague, Czech Republic). The mutant strain BM71, deficient in the expression of surface-associated M and/or M-like proteins, was generated from the wild-type serotype as described previously [42]. From a culture of *S. pyogenes* on blood Todd-Hewitt agar (API) or Todd-Hewitt agar (BM71), a few colonies were transferred to Todd-Hewitt broth at 37°C in 5% CO₂. For BM71, 5 µg ml⁻¹ tetracycline was added to the culture medium. After an overnight growth at 37°C, the bacteria were harvested and washed three times in phosphate-buffered saline (PBS) containing 116 mM NaCl, 1.7 mM KH₂PO₄, and 4.9 mM Na₂HPO₄. Bacterial suspensions were adjusted (photometrically optical density of 620) to the desired concentration. The viability of the bacteria (>95% viability) was verified using a live-dead kit from Molecular Probes. For some experiments, bacteria were killed by heating in a water bath at 80°C for 10 min.
for 30 min with ALEXA Fluor® 594 F(ab’)_2 fragment of goat anti-rabbit IgG, final dilution of 1/1500.

Glass coverslips were washed with methanol and overlaid with 0.25 ml poly L-lysine (0.2 mg ml⁻¹ in water). After evaporating the added fluid at 50–65°C, the coverslips were washed twice with distilled water. After a final wash with PBS, the fixed and stained cells were adhered to the poly-L-lysine-coated coverslips, and the samples were mounted using Dako-mounting medium.

Visual inspection and recording of images were performed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled charged-coupled device camera, using a Plan Apochromat 100× oil immersion objective. Images were acquired and handled using Image Pro Plus and Adobe Photoshop 7.0.

**Spectrofluorometry**

To quantify the amount of Lucifer Yellow internalized by phagocytosing neutrophils, samples were washed twice after experimentation and were then resuspended in Na medium containing 0.1% Triton X-100 to lyse the cells. Cell components not solubilized by Triton were removed by centrifugation at 16,100 g for 1 min prior to analysis of the supernatants (200 μl, room temperature) in a FLUostar Galaxy spectrofluorometer (BMG Labtechnologies, Germany) with λ_ex = 410 nm and λ_em = 520 nm.

**Colocalization assay**

Pinosomal reuptake of azurophilic granule marker was investigated after a synchronized interaction of neutrophils with IgG-opsonized zymosan (two particles/cell). After a 1-min incubation at 37°C, the cells were put on ice, and exocyted CD63 was immunostained during a 10-min incubation with rabbit anti-human LAMP-3 antibody, followed by a 10-min incubation with ALEXA Fluor® 594 F(ab’)_2 fragment of goat anti-rabbit IgG. After extensive wash, Lucifer Yellow was added, and pinocytosis was allowed at 37°C for 1 min. After wash, the samples were then fixed using 2% PFA and mounted onto poly-L-lysine coverslips. Images were recorded as described above.

**RESULTS**

FcγR-mediated phagocytosis of opsonized zymosan or latex beads has been shown to stimulate a localized pinocytic response in human neutrophils [24]. This pinocytic response was suggested to be a mechanism compensating for an increase in membrane area caused by the secretion of granules toward a phagocytic prey. Phagosomal delivery of granule content is important for killing of microorganisms, and therefore, we wanted to investigate whether induction of pinocytosis was also observed when neutrophils were stimulated with bacteria. 

Previously, we have shown (L. Staali et al., unpublished results; [40]) that the S. pyogenes mutant BMJ71 is avidly phagocytosed by human neutrophils; can trigger an oxidative burst and a rapid secretion of granule markers to the plasma membrane; can stimulate the fusion of specific and azurophilic granules with phagosomes; and is killed and degraded in phagosomes. Therefore, the BMJ71 bacterium was used as a Gram-positive, model bacterium. As can be observed in Figure 1, A–C, a 1-min pulse of the fluid-phase marker Lucifer Yellow, added 5 min after allowing phagocytosis, resulted in an avid uptake of the probe. The fluorescent signal partly colocalized with some of the bacteria (Fig. 1B; cf., Fig. 1C), but a punctuate staining of small structures in close vicinity to the internalized bacteria was also evident. It should be observed that the rapid uptake of Lucifer Yellow is restricted to localization to the part of the neutrophil that is engaged in phagocytosis. Of note, unstimulated neutrophils have a very low level of spontaneous pinocytic activity (not shown).

Next, nonopsonized latex beads (Fig. 1, D–F) and zymosan particles (not shown) were used to determine whether the observed pinosome formation is a response occurring during phagocytosis of any particle. As shown in Figure 1, D–E, the interaction of neutrophils with latex beads did not efficiently trigger pinosome formation. Occasionally, beads (Fig. 1F) or zymosan particles (not shown) were surrounded by a rim of Lucifer Yellow, but very few punctuate structures were observed.

The results shown in Figure 1 indicate that a pinocytic response can be induced by nonopsonized BMJ71 bacteria and that pinosome formation is not a common sequel to the phagocytic uptake of any particle. Therefore, the signaling requirements for pinosome formation triggered by bacteria were investigated.

An increase of the intracellular calcium level has been shown earlier to stimulate pinocytosis in neutrophils [24]. As shown in Figure 2, A and B, ionomycin-induced pinocytic activity was not localized to a particular region of the neutrophil, a finding that correlates with the diffuse pattern of secretion of granules triggered by this ionophore [24]. When preincubating the cells with cytochalasin D, a cell-permeable fungal toxin, which inhibits actin polymerization by capping the barbed end of actin filaments [43], ionomycin-induced pinocytosis was inhibited (Fig. 2, C and D). This indicates a role for the actin cytoskeleton in pinosome formation induced by an artificial elevation of cell calcium.

In contrast, the pinocytic uptake of Lucifer Yellow induced by BMJ71 bacteria also occurred after a preincubation with cytochalasin D (Fig. 3, A–C). This observation indicates that internalization of bacteria is not necessary for the pinocytic response, as the phagocytosis of these bacteria is almost completely inhibited by cytochalasin D [40]. The relative insensi-
Previous studies have shown that there is a difference in survival of phagocytosed bacteria when comparing the streptococcal wild-type AP1 strain with the M protein-deficient, mutated variant BMJ71 [40]. Under the conditions of our assay, both strains are efficiently phagocytosed by human neutrophils [40]. Furthermore, the AP1 bacteria have been shown to selectively inhibit the fusion between azurophilic granules and phagosomes in neutrophils (L. Staali et al., unpublished results). We therefore wanted to investigate if AP1 bacteria were less efficient in stimulating pinosome formation than BMJ71 bacteria. This would support the previously suggested link between azurophilic granule secretion and pinosome formation [24].

In experiments comparing pinosome induction by BMJ71 and AP1 bacteria, we initially used the fixable membrane marker FM* 1–43FX. This probe is fluorescent only when bound to membrane and rapidly labels the plasma membrane in a reversible manner, allowing probe that has not been internalized to be efficiently removed by a single wash. In neutrophils interacting with BMJ71 bacteria, abundant FM* activity of bacteria-induced pinocytosis to cytochalasin treatment is in agreement with earlier data about pinosome formation induced by IgG-opsonized beads [24]. Furthermore, our results suggest mechanistic differences and a different involvement of the actin filament network in calcium- and bacteria-induced pinosome formation.

Next, we investigated whether calcium-free conditions impaired the uptake of Lucifer Yellow triggered by bacteria. Of note, the depletion protocol used did not adversely affect the interaction between neutrophils and bacteria [at a bacteria/cell ratio of 10:1, 66.7% (calcium-depleted), 62.5% (control) of the neutrophils interacted with bacteria (on average, 7.3 and 7.9 bacteria/cell, respectively)]. As shown in Figure 3, D–F, no pinosome formation was triggered by bacteria when intracellular calcium was chelated by BAPTA. Sensitivity to such treatment has been observed earlier for neutrophil secretion of granules [7].

Inhibitors of phosphatidylinositol (PI)-3 kinase block several steps in endocytic traffic and inhibit phagocytosis of large particles (however less efficiently that of small particles) [44]. PI(3) phosphate production is also important for phagosomal acquisition of late endocytic markers and the recruitment of EEA1 to membranes [45]. Hence, the sensitivity of bacteria-induced pinocytosis to wortmannin was investigated, after verifying that this drug did not inhibit the interaction between neutrophils and bacteria [at a bacteria/cell ratio of 10:1, 66.7% of the wortmannin-treated neutrophils interacted with bacteria (on average, 7.6 bacteria/cell)]. As can be observed in Figure 3, G–I, pinosome formation also occurred after wortmannin treatment.
1-43FX fluorescence was observed in phagosomes as well as in pinosomal structures (Fig. 4, A–C; note that part of the signal in Figure 4C can be ascribed to bleed-through of FM\textsuperscript{1-43FX}). These pinosomal structures were even more prominent if more than one cell was engaged in frustrated phagocytosis of the same cluster of bacteria (not shown). A strikingly different staining pattern was observed in neutrophils stimulated by AP1 bacteria (Fig. 4, D–F). In these cells, FM\textsuperscript{1-43FX} was only occasionally observed in phagosomes, and the staining was not found in pinosomal structures. The different staining patterns were blindly evaluated by three persons, who each selected 20 single cells containing two to five red-stained bacteria and then changed the filter to observe whether the phagosomes exhibited green FM\textsuperscript{1-43FX} fluorescence. Sixty-five percent of the neutrophils containing BMJ71 bacteria exhibited green fluorescence, whereas this was the case for only 19% of the AP1-containing cells.

To quantify fluid-phase uptake in neutrophils exposed to AP1 and BMJ71 bacteria, the amount of Lucifer Yellow recovered in cell lysates was measured by spectrofluorometry (Fig. 5). Uptake of Lucifer Yellow was analyzed after a 1-min pulse of the fluid-phase probe, added 10 min (A) or 1 min (B) after presenting the bacteria to the neutrophils. Although both bacterial strains interacted to a similar extent with the neutrophils, the ability of AP1 bacteria to induce an uptake of Lucifer Yellow was clearly attenuated, as compared with the mutant bacteria.

It should be noted that when a prolonged chase period was allowed between phagocytosis and the addition of the fluid-phase marker, uptake of Lucifer Yellow into pinosomes and phagosomes occurred (not shown). This indicates that after the completion of phagocytosis, bacteria-stimulated pinosome formation continues. Also, Lucifer Yellow did appear in phagosomes even when such a chase period was performed in the presence of cytochalasin D. These data indicate that formed

![Fig. 4. Effect of BMJ71 and AP1 bacteria on neutrophil membrane internalization.](image)

![Fig. 5. Quantification of neutrophil fluid-phase uptake and efflux stimulated by BMJ71 and AP1 bacteria.](image)
pinosomes can intermingle with a preformed phagosomal compartment, at least during the first 10–15 min after phagosome formation.

Next, neutrophils incubated with AP1 or BMJ71 bacteria were loaded with Lucifer Yellow 1 min after allowing phagocytosis, and the efflux of the probe was subsequently studied at 37°C (Fig. 5C; cf., Fig. 5B). As shown in this figure, Lucifer Yellow, preloaded into pinosomal and/or phagosomal compartments, was released from the cells during a 10-min chase at 37°C.

It has been proposed that the pathogenicity of *S. pyogenes* is related to the coexpression of M and M-like proteins on the bacterial surface. However, when live and heat-killed bacteria were compared, the heat-killed API strain was attenuated in its ability to inhibit fusion between phagosomes and azurophilic granules (L. Staali et al., unpublished results). This indicates that factors other than M and M-like proteins could be important for modulating granule fusion, or alternatively, the structural integrity of the M proteins are compromised by the heat-killing of bacteria. Nevertheless, again, suggestive of a causative link between azurophilic granule secretion and pinosome formation, an enhanced uptake of Lucifer Yellow by neutrophils incubated with dead AP1 bacteria was observed, as shown in Figure 6. Approximately half as many cells interacting with live AP1 bacteria were scored as positive, as compared with cells interacting with heat-killed bacteria, when cells with two to 10 bacteria were blindly scored as negative, intermediate, or clearly positive for Lucifer Yellow uptake (data not shown). It is important that control experiments showed that live and heat-killed API bacteria were phagocytosed equally well under the conditions of these experiments. This was demonstrated by fluorescence microscopy, using Oregon green-labeled bacteria (all bacteria green) and immunostaining of the extracellular bacteria (red).

The suggested coupling between azurophilic granule secretion and pinosome formation was then investigated by performing an experiment where exocytosed CD63 was immunostained, prior to allowing a 1-min chase period at 37°C in the presence of Lucifer Yellow. As can be observed in Figure 7, immunostained CD63 was focally internalized, and it is important that it was, to a high degree, colocalized with internalized Lucifer Yellow.

**DISCUSSION**

In the human neutrophil, the formation of a bactericidal and acidic phagosome is a complex process that involves its sequential acquisition of components (membrane and content) from various endosomal and granular compartments. This process is regulated by interactions of the progressively changing nascent phagosomal membrane with various cytosolic and cytoskeletal proteins. Apart from granules, fusion events with early phagosomes could involve early endosomal compartments, whereas late phagosomes would be expected to preferentially interact with late endosomal compartments. These interactions are dependent on phosphoinositide metabolites and the recruitment of various Rab proteins [10].

Early endosomal vesicle-associated membrane protein-positive compartments have been demonstrated to fuse with the plasma membrane at the site where a phagosome is formed and are considered to aid phagocytosis by providing necessary membrane. The rapid triggering of fluid-phase pinocytosis in the human neutrophil by IgG-coated model particles [24] and *S. pyogenes* bacteria (this paper) would seem to contradict this model, as it implies that membrane, rather, is removed from the site of phagosome formation. However, it is possible that the massive granule exocytosis occurring during human neutrophil phagosome formation [7] makes this professional phagocyte, containing many readily releasable membrane pools, a special case.

The pinocytic activity is relatively low in resting neutrophils. However, upon stimulation of these cells with opsonized particles, the amount of pinosomes increases drastically, and the pinosomes can be observed to be localized to the site of cell/particle interaction [24]. Also nonopsonized BMJ71 bacteria trigger a localized pinocytic response, but this is not the case for nonopsonized model particles, indicating that pinosome formation is not an inherent feature of phagocytosis. This is also indicated by the persistence of pinosome formation, although phagocytosis is blocked by cytochalasins (ref. [24]; present study). Rather, pinocytic activity is triggered by specific receptor ligation and clustering, which in the case of BMJ71, could involve bacterial structures such as lipoteichoic acid and peptidoglycans interacting with Toll-like receptors or other neutrophil receptors.

Ionomycin has been demonstrated to be an effective inducer of pinocytosis, and cross-linking of various receptors can trigger an elevation of cytosolic calcium. However, ionomycin-induced pinosome formation is not localized and furthermore, was shown to be almost completely blocked by pretreatment of the cells with cytochalasin D. This indicates that the ionomycin-induced pinocytosis process is highly dependent on actin polymerization. In contrast, the localized pinocytic response to nonopsonized bacteria was only moderately affected by cyto-
fuse. In the case of the phagosome, the pinosomal traf-
membrane, locations where azurophilic granules dock and
occur at the plasma membrane and at the phagosomal
fusion of azurophilic granules with a target membrane is linked
both processes to a depletion of intracellular calcium stores. If
suggested coupling is further substantiated by the sensitivity of
these processes to actin disruption imply a causal link. This
granule secretion and pinosome formation and the insensitivity
of membrane area of cells and/or phagosomes, as the secretion
that the formation of pinosomes is important for the regulation
localized elevation of cytosolic calcium in the vicinity of phago-
somes [47] have been suggested to be important for remodeling
the actin mesh surrounding newly formed phagosomes and for
signaling to the fusion of granules with phagosomes. It is tempting
to speculate that phagosomal delivery of fluid-phase
by pinosomes (high calcium concentration) could be one mech-
anism, whereby a high concentration of cytosolic calcium in
the vicinity of phagosomes is maintained.

When comparing the pinocytic activity of the wild-type AP1
strain with that of the mutant BMJ71, lacking the surface
proteins M1 and H, a difference was observed. Pinosomes were
much more frequent and prominent in neutrophils stimulated
by BMJ71. The mechanisms by which S. pyogenes bacteria
resist killing are not yet understood, although great importance
has often been ascribed to an inhibition of the phagocytosis
process mediated by M and/or M-like proteins [38, 39]. How-
ever, it was [40] recently pointed out that the traditional
bactericidal assay only measures bacterial survival and that an
inhibition of phagocytosis has to be demonstrated by other
methods. Using a variety of techniques, we could demonstrate
that S. pyogenes bacteria of the M1 serotype, expressing M and
M-like proteins, were readily phagocytosed by human neutro-
phils and were able to survive and multiply inside phagosomes.
Based on these findings, it was suggested that proteins M1 and
H were required for the intracellular survival of virulent S.
pyogenes bacteria. Moreover, these bacteria selectively inhibit
phagosomal maturation by preventing the fusion of azurophilic
granules with phagosomes (L. Staali et al., unpublished re-
results). Inhibition of this host defense mechanism, normally
triggered by phagocytosis, may contribute to the intracellular
survival of this pathogen and the recurrence of S. pyogenes
infections.

For other pathogens, inhibition of phagosome-lysosome fusi-
ion has been shown to be associated with the presence of
particular surface molecules on the pathogen or on the phago-
osomal membrane. For example, in the case of Leishmania,
maturatin inhibition requires lipophosphoglycan expression at
the parasite surface [48]. In the case of mycobacteria, inhibi-
tion of phagosome-lysosome fusion has been suggested to be
mediated by the presence of the tryptophane aspartate-contain-
ing coat host protein, which can be retained on the phagosomal
membrane if clumps of viable bacteria are internalized [49,
50]. Moreover, by reconstitution of phagosome-lysosome fusion
in vitro, it has been shown that the inhibition of phagosome-
lysosome fusion caused by mycobacteria is regulated by solu-

Fig. 7. Pinosomal reuptake of azurophilic granule marker. After a synchro-
nized interaction (two particles/cell), neutrophils were allowed to phagocy-tose
IgG-opsonized zymosan at 37°C for 1 min. Thereafter, the cells were put on ice,
and exocyted CD63 was immunostained during a 20-min incubation. After
extensive wash, Lucifer Yellow was added, and pinocytosis was allowed at
37°C for 1 min. CD63 staining is shown (C), whereas Lucifer Yellow fluores-
cence is shown (D). The corresponding DIC image is shown (A). (B) A color
overlay of CD63 and Lucifer Yellow staining is shown. Original size bar = 10
μm.

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ble factors that are secreted by live bacteria or that are present on the phagosome membrane [51, 52].

Professional phagocytes have evolved a number of mechanisms, such as the acidification of newly formed phagosomes, the oxidative burst, and the fusion of phagosomes with various granules to kill invading pathogens [2, 3]. As the mutant strain BMJ71 lacks the M1 protein and protein H on its surface, it is tempting to speculate that these proteins are essential for protection of the bacteria from the postphagocytic, antibacterial defenses encountered inside the human neutrophils. The results of this study suggest that the neutrophil membrane transport processes normally triggered during phagocytosis can be affected and disturbed by the presence of S. pyogenes M1 protein and protein H and possibly other factors sensitive to heat-killing of the bacteria. A suggested causal relationship between azurophilic granule secretion and pinosome formation was substantiated by the observation that these events were specifically suppressed in live AP1 bacteria and that heat-killing of the bacteria partially reversed both responses (L. Staali et al., unpublished results; present study). The early pinocytic response studied here could be important for subsequent membrane traffic events and for the differences in survival observed for the two bacterial strains.

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