Phagocytosis and killing of microbial pathogens by professional phagocytes is an essential component of the innate immune response. Recently, heterologous transfection of individual receptors into nonmyeloid cells has been used successfully to elucidate the early steps that signal phagosome formation. It is unclear, however, whether the vacuoles formed by such transfected cells are bona fide phagosomes, capable of fusion with endomembranes, of luminal acidification, and of controlling the growth of microorganisms. The aim of the current study was to determine whether COS-1 and Chinese hamster ovary cells, rendered phagocytic by expression of human FcγRIIA receptors, express the cellular machinery required to support phagosomal maturation. Immunolocalization studies demonstrated that early endosomes, as well as late endosomes and/or lysosomes, fuse sequentially with phagosomes in the transfectedants. Microfluorescence ratio imaging of particles labeled with pH-sensitive dyes revealed that maturation of the phagosome was accompanied by luminal acidification. The drop in pH, which attained levels comparable to those reported in professional phagocytes, was prevented by inhibitors of vacuolar-type H^+^-ATPases. Optimal phagosomal acidification required elevation of cytosolic [Ca^{2+}], suggesting that it results from fusion of endomembranes bearing proton pumps. Moreover, the transfected cells effectively internalized live bacteria. Opsonization was essential for bacterial internalization, implying that it occurred by FcγRIIA-mediated phagocytosis, as opposed to invasion. Uptake into phagolysosomes was associated with inhibition of bacterial growth, due at least in part to the low intraphagosomal pH. These studies indicate that the biochemical events that follow receptor-mediated particle internalization in cells transfected with FcγRIIA receptors closely resemble the process of phagosomal maturation in neutrophils and macrophages. FcγRIIA-transfected cells can, therefore, be used as a model for the study of additional aspects of phagocyte biology.

Phagocytosis of invading microorganisms by leukocytes represents a vital component of host defense against infection (1–3). Microbes are taken up into a phagocytic vacuole, which matures upon fusion with endomembrane compartments including endosomes and lysosomes (4–6). Transfer of soluble and membrane-associated components from these compartments confers microbicidal activity to the phagosome (7).

The initial steps of the phagocytic sequence involve recognition and binding of microbial pathogens, a process enhanced by soluble components of serum, including complement fragments and immunoglobulins. These serum factors, known as opsonins, are recognized by receptors on the phagocyte cell membrane, such as the complement receptor 3 and immunoglobulin Fc receptors. Recent studies have begun to elucidate the signaling pathways involved in this complex series of events (for reviews, see Refs. 3 and 8–10). Binding of antibody-opsonized particles to the phagocyte surface initiates activation of nonreceptor tyrosine kinases of the Src family and of p72^Syk^ that are important for the phosphorylation of tyrosine residues within the Fc receptor complex (11, 12). Spatial grouping (“clustering”) of p72^Syk^ appears to be a key step in the early phase of phagocytosis (13) and is accompanied by an accumulation of additional phosphorylated proteins in the vicinity of the phagocytic particle, resulting in activation of Ser/Thr (14, 15) and phosphoinositide kinases (16, 17), and a rise in free cytoplasmic Ca^{2+} (18–20). These events are associated with the formation of an actin-rich cup encircling the nascent phagosome, leading to internalization of the particle (3, 21–23).

Definition of the detailed molecular mechanisms triggered by individual receptors is complicated by the co-expression of multiple receptor types in phagocytes. Not only are both complement and Fc receptors present in neutrophils and macrophages, but several different members of the Fc receptor family co-exist in these cells. To overcome this difficulty, several laboratories have opted to express individual, well-defined receptors in nonphagocytic cells, which are devoid of endogenous opsonin receptors (10, 13, 17). Remarkably, heterologous expression of single subtypes of Fc receptors, such as the FcγRIIA receptor, suffices to induce effective internalization of IgG-opsonized particles (10).

Such heterologous models are attractive not only because they afford analysis of individual receptors but also because the cells used for expression are more amenable to transfection than natural phagocytes. This facilitates the introduction of cDNAs encoding other molecules of interest, and this model can be used to study multiple aspects of phagosomal formation, maturation, and bactericidal activity. To date, however, the use of heterologous expression systems has been limited to the
analysis of the early steps in the signaling cascade (10, 20, 24). This is due, in part, to the fact that the fate of phagosomes in cells transfected with opsonin receptors is not known. It is not clear whether such phagosomes undergo a maturation process that resembles that reported for professional phagocytes.

The aim of the current study was to determine whether nonmyeloid cells, rendered phagocytic by expression of Fc receptors, possess the cellular machinery required for phagosomal maturation. Immunofluorescence was used to monitor the interaction between endomembrane compartments and the nascent phagosome in COS-1 and Chinese hamster ovary (CHO) cells transfected with human FcRIIa. In addition, microfluorescence ratio imaging of zymosan particles labeled with pH-sensitive dyes was used to study the development of phagosomal acidification.

**EXPERIMENTAL PROCEDURES**

**Materials and Solutions—**EGTA, Hepes, ATP, zymosan, and RPMI 1640 medium were obtained from Sigma. Albumin was obtained from Calbiochem (La Jolla, CA). Nigerosin, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate-transferrin (Tfn), the succinimidyld ester of Oregon Green 488, Texas Red-labeled zymosan, Lucifer Yellow, and the acetoxyethyl esters of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), Fura-2, and 1,2-bis(aminophenox)-ethane-N,N′,N″,N‴-tetracetic acid (BAPTA) were all from Molecular Probes (Eugene, OR).

Bicarbonate-free RPMI 1640 medium was buffered to pH 7.3 with 25 mM Na-Hepes. The sodium-rich medium used for incubation of intact cells contained 140 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM Hepes (pH 7.4). PBS consisted of 140 mM NaCl, 10 mM KCl, 8 mM sodium phosphate, 2 mM potassium phosphate, pH 7.4. The Na⁺-rich medium used in the Na⁺ loading experiments contained 140 mM NaCl, 5 mM glucose, 15 mM Hepes, pH 7.4. The Na⁺-free medium was identical except that Na⁺ was substituted by N-methyl-D-glucamide. The K⁺-rich medium had the same composition as Na⁺ medium, but NaCl was replaced by KCl. In all cases, the osmolarity of the medium was prepared to 290 ± 5 mosm with the major salt. Dulbecco's modified Eagle's medium was obtained from Life Technologies, Inc. Fetal bovine serum was obtained from Life Technologies, Inc. and heat-inactivated by incubation at 56 °C for 30 min prior to use in cell culture.

Monoclonal antibodies to lysosome-associated membrane protein 1 (LAMP-1) and LAMP-2 were obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa and Johns Hopkins University School of Medicine (Baltimore, MD). Mouse monoclonal antibodies against CD63 were generously provided by Dr. J. Bonifacino (National Institutes of Health, Bethesda, MD). Transfected CHO cells with the human Tfn receptor was accomplished using the polyethylenimine (PEI) transfection reagent (Fugene 6 [Roche Molecular Biochemicals]) as instructed by the manufacturer. To study endosome-phagosome fusion, cells transfected with the human Tfn receptor were serum-starved for 1 h in Dulbecco's modified Eagle's medium and then allowed to internalize opsonized RBC for 15 min. Extracellular RBC were then lysed by hypotonic shock with water, and the cells were subsequently incubated with 50 μM tetramethylrhodamine isothiocyanate-labeled Tfn for 10 min. The cells were then cooled to preclude further membrane traffic and observed with epifluorescence and Nomarski optics.

To monitor the kinetics of endosome-phagosome fusion, the cells were first labeled with tetramethylrhodamine isothiocyanate-Tfn as above, chased for 7 min to clear surface-bound Tfn, and then allowed to internalize opsonized RBC for 10 min. The cells were then incubated further for periods of up to 30 min and analyzed.

**Measurement of Cytosolic Calcium ([Ca²⁺]ᵢ)—**To measure cytosolic free calcium, the cells were loaded with fura-2 by incubation with 2 μM of the precursor ester for 20 min at 37 °C. Fura-2 ratio fluorescence measurements were performed on a Nikon Diaphot TMD microscope (Nikon Canada, Toronto, Ontario, Canada) equipped with a 100-W xenon lamp, a shutter/rotating mirror/fiber optic assembly (RatioMaster, Photon Technologies Inc., South Brunswick, NJ), a Fluor × 40/1.3 oil-immersion objective, and a high sensitivity photometer (D-104, PTI) interfaced to a Dell Pentium computer via a 12-bit A/D board (Labmaster, National Instruments). The cells were alternately excited at 340 and 380 nm while the emission at 510 nm was recorded. Photometric data were acquired at 10 Hz using the Felix software (PTI). The microscope was equipped with a separate light source for long wavelength transillumination (λ>620 nm), a 580-nm emission dichroic mirror, and a separate video camera (MTI 72, Dage-MTI, Michigan City, IN), to allow continuous Hoffmann-enhanced visualization of the cells during the fluorescence measurements. Calibration was performed as described previously (26). Where indicated, cells were also loaded with 10 μM BAPTA (the ester form of BAPTA) by incubation for 20 min at 37 °C.

**Measurement of Cytosolic pH ([pH]ᵢ)—**To measure pH, COS-2A cells were grown to 60–70% confluence on 25-mm coverslips and loaded with 2 μM of the ester precursor of BCECF for 20 min at 37 °C. Fluorescence of BCECF was measured essentially as described previously using the PTI system detailed above, with the filter combination described earlier (27). Acute acid loading was accomplished by the ammonium prepulse technique (28). Calibration of the fluorescence ratio versus pH was performed for each experiment by equilibrating the cells in isotonic K⁺-rich medium buffered to varying pH values (between 7.45 and 6.0) in the presence of the K⁺-inorganic phosphate nigericin (5 μM). Calibration curves were constructed by plotting the extracellular pH, assumed to be identical to the cytosolic pH under these conditions, against the corresponding fluorescence ratio (29).

**Measurement of Phagosomal pH ([pH]ᵢ)—**For these studies, zymosan particles were labeled with FITC alone or in combination with Oregon Green, as indicated. COS-2A or CHO-2A cells plated onto 25-mm coverslips and grown to 70–80% confluence were overlayed with opsonized, fluorescently labeled zymosan particles (~1 × 10⁵/coverslip) and allowed to interact for 1 h. The coverslip was placed in a thermostatted Leiden holder on the stage of a Zeiss IM-35 microscope with a × 63, 1.4 numerical aperture oil-immersion objective. Measurements of pHᵢ were obtained through the combined application of video microscopy and fluorescence ratio imaging, as described previously (29). Calibration of fluorescence ratio versus pHᵢ was obtained by fixation of cells with 1.6% paraformaldehyde for 20 s followed by permeabilization with 0.2% Triton for 1 min. Preliminary experiments indicated that treatment with 1% paraformaldehyde did not affect the fluorescent properties or pH sensitivity of the probe. After washing, the pH of the bathing medium was varied, and the corresponding fluorescence ratio was recorded.

**Immunofluorescence Microscopy—**Immunofluorescence studies were performed essentially as described (26). For localization of the lysosomal markers CD63, LAMP-1, and LAMP-2, cells (with or without opsonized zymosan treatment) were fixed with cold methanol (~20 °C) for
mental Procedures.”

15 min. The cells were then washed with ice-cold PBS and labeled with primary antibody for 2 h at room temperature or overnight at 4 °C. The cells were then washed with PBS and labeled with Cy3-labeled secondary for 1 h at room temperature, washed, and mounted using Slow Fade (Molecular Probes, Eugene, OR).

RESULTS

Assessment of pHr—To examine the molecular events involved in the intracellular processing of microbes in nonprofessional phagocytic cells, we utilized COS-1 and CHO cells stably expressing the human FcγRIIA receptor (COS-2A and CHO-2A, respectively) as model systems. These cells have been shown to be capable of internalizing IgG-opsonized particles, such as sheep RBC (25, 30).

The plasma membrane of most mammalian cells, including leukocytes, is virtually devoid of vacuolar (V-type) proton pumps or ATPases. In phagocytes, acidification of the phagosomal lumen requires the insertion of V-ATPases through fusion with endosomes and/or lysosomes. Therefore, as an initial approach to establish whether phagosomal maturation occurs in COS-2A and CHO-2A cells, we measured pHr. We utilized microfluorescence ratio imaging of yeast particles (zymosan) labeled with pH-sensitive fluorescent dyes. Most previous studies of pHr have utilized particles covalently labeled with fluorescein. Although this probe has a large quantum yield and exquisite pH sensitivity, its pK1′ (∼6.5) limits the measurements to the moderately acidic range. As illustrated in Fig. 1 (triangles), the responsiveness of this dye below pH 5.5 is marginal. Preliminary observations using FITC-labeled yeast revealed that the acidification of the phagosome might exceed the limit of sensitivity of this probe. To extend the range of sensitivity of our determinations, we used a second probe, namely Oregon Green 488 (pK1′ ∼ 4.7). Following covalent attachment to zymosan particles, the emission of this dye is linearly sensitive to pH in the 4.0–5.5 interval (Fig. 1, crosses), complementing the range of sensitivity of fluorescein. Therefore, for all subsequent experiments, we used particles that were dual-labeled with FITC and with the succinimidyl ester of Oregon Green.

Fig. 1 illustrates an experiment in which particles were added to the cells during the course of the observation, in order to capture the development of the acidification process. As illustrated by the triangles, acidification was complete within 5 min, maintained for at least 20 min, and recovered readily from the addition of NH4 +. Similar results were obtained using CHO-2A cells (not illustrated).

Role of the V-ATPase in Acidification—Phagosomal acidification in professional phagocytes is mediated largely by V-ATPases (31, 32). To ascertain whether a similar mechanism is involved in the heterologous transfectants, COS-2A cells were allowed to internalize labeled zymosan and then treated with concanamycin, a potent and selective inhibitor of V-ATPases (33). As before, the phagosomal location of the particle was first verified (Fig. 3A), and the inhibitor was then added. As shown in Fig. 3A, concanamycin triggered the gradual dissipation of the phagosomal acidification. The slow course of alkalinization, which was not accelerated by using higher doses of the inhibitor, implies that the phagosomal membrane is comparatively tight to H+ equivalents. This contrasts with the faster dissipation rates observed in endosomes (34) and is compatible with the markedly more acidic pH of phagosomes, which approached 4.5 in some experiments (e.g., Fig. 3B). A similar rate and extent of dissipation was recorded using bafilomycin (Fig. 3B), a different macrolide antibiotic also reported to inhibit V-ATPases selectively (35). When added prior to phagocytosis, the inhibitors precluded phagosomal acidification (not shown). Neither bafilomycin nor concanamycin affected the fluorescence of extracellular particles. Jointly, these experiments confirm that the marked acidification of the phagosomal compartment of COS-2A (and also CHO-2A cells; not illustrated) is mediated by V-ATPases, as is the case in professional phagocytes.

Whereas most mammalian cells do not express V-ATPases in their plasmalemma, active proton pumping has been reported in the surface membrane of osteoclasts and in certain epithelia, including the renal proximal tubule. Because COS-2A cells are derived from the kidney, and because the acidification of the phagosome developed rapidly, it was important to consider whether V-ATPases are constitutively present and active in the plasma membrane of these cells. To this end, the cytosolic pH of COS-2A cells was measured with the indicator dye BCECF. Neither bafilomycin (Fig. 4) nor concanamycin affected the basal pH of COS-2A cells. To magnify the possible activity of V-ATPases, thereby facilitating detection, the cells were acid-
endosomes, CHO-2A cells were allowed to take up opsonized zymosan particles. The location of internal (open arrowheads) and external (closed arrowheads) particles, identified as described in the text, is indicated. B, pseudocolor image of the fluorescence ratio (proportional to pH; see calibration bar below) of the zymosan particles, recorded in cells bathed in medium of pH 7.4. C, fluorescence ratio of the zymosan particles immediately after addition of 40 mM NH₄Cl. D, time course of the pH changes experienced by extracellular (circles) and internalized (squares) particles. Where indicated, the external pH was reduced from 7.4 to 6.0 or 40 mM NH₄Cl was added to the medium at pH 7.4. E, acidification of a zymosan particle upon internalization (triangles). For comparison, the pH of a particle that remains external throughout is illustrated. The occurrence of internalization was tested by addition of 40 mM NH₄Cl.

To assess the interaction between phagosomes and early endosomes, CHO-2A cells were allowed to take up opsonized RBC and, when phagocytosis was completed, Tfn was added. After 10 min, some of the phagosomal membranes were lined by Tfn receptors (Fig. 5C), indicating fusion of early endosomes with the phagosome. Not all the RBC were surrounded by labeled membranes, which may reflect asynchrony in the internalization and fusion process. Note that in these experiments phagocytosis preceded binding of Tfn, ruling out internalization of surface Tfn along with the nascent phagosome. These data indicate that at least a fraction of the phagosomes formed are capable of fusing with early endosomes. Similar results were obtained in COS-2A cells using zymosan particles or when pulsing with Tfn and a 7-min chase preceded phagocytosis (not shown).

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The next stage in the maturation of the phagosome in professional phagocytes is fusion with late endosomes and ultimately with lysosomes. This event was analyzed next. Because some of the markers proteins such as the LAMPs are expressed in both late endosomes and lysosomes, no effort was made in our studies to distinguish between these compartments. The distribution of three markers was compared in cells before and after exposure to zymosan. As shown in Fig. 6, CD63, LAMP-1, and LAMP-2 all display a random punctate distribution in untreated COS-2A cells (Fig. 6, A, D, and G, respectively). Following internalization of zymosan particles, which were labeled with a different fluorophore, fusion was monitored (Fig. 6, C, F, and I), some of the endolysosomal markers were mobilized and fused with the phagosomal membrane (Fig. 6, B, E, and H). Note that only a fraction of the adherent zymosan particles became internalized (solid arrowheads). These findings of spatial co-localization indicate that late endosomes and/or lysosomes also fuse with phagosomes in cells heterologously transfected with the FcγRIIA receptor.
Phagosomal Maturation in Fc Receptor-transfected Cells

The sequential nature of the fusion of endosomes and lysosomes was analyzed in detail. Early endosomes were loaded with Tf and, after chasing the surface-bound Tf, phagocytosis was initiated by exposing the CHO cells to opsonized RBC for 15 min. After varying periods of time, the reaction was stopped, and the presence of Tf-bound and LAMP-1-positive phagosomes was assessed as above. The results of three similar experiments are summarized in Fig. 7. Tf-bound phagosomes were LAMP-1-positive 30 min after phagocytosis; consequently, the fraction of phagosomes stained for LAMP-1 is modest after 15 min but increases progressively. Most of the phagosomes were LAMP-1-positive 30 min after phagocytosis was completed. These results bear remarkable resemblance to the course of phagosomal maturation in monocytes/macrophages (see, for example, Ref. 5).

Role of Calcium in Phagosomal Acidification—There is disagreement concerning the requirement for calcium in the process of phagosomal maturation. In neutrophils, fusion of phagosomes with lysosomal like granules that express CD63 is thought to be strictly dependent on elevated [Ca\(^{2+}\)] (19). By contrast, phagosome to lysosome fusion was unaffected when [Ca\(^{2+}\)] transients were prevented in macrophages (37). This discrepancy may reflect differences between neutrophils and macrophages in the regulation of phagosome to lysosome fusion and from the fact that multiple and different receptors are activated during phagocytosis. We therefore used the heterologous transfection system to reanalyze the [Ca\(^{2+}\)] dependence of phagosomal maturation.

As reported earlier (20), engagement of FcRIIA induced a rapid and transient elevation of [Ca\(^{2+}\)] in the FcRIIA transfectants (Fig. 8A). Suspension of the cells in Ca\(^{2+}\)-free medium, followed by depletion of the intracellular stores using thapsigargin, abrogated the [Ca\(^{2+}\)] response to a subsequent addition of IgG-opsonized particles (Fig. 8B). The response to receptor cross-linking was similarly eliminated by preloading the cells with the chelating agent BAPTA (Fig. 8C). Under these conditions, particle internalization was unaffected, as reported for professional phagocytes (38). Calcium independence of phagocytosis is also in accord with the observation by Odin et al. (20) that different regions of the FcRIIA mediated calcium flux and particle internalization.

BAPTA was applied in parallel experiments to assess phagosomal acidification, used as an index of maturation. As shown in Fig. 8D, chelation of intracellular Ca\(^{2+}\) diminished but did not prevent the development of phagosomal acidification. Phagosomal pH averaged 4.7 ± 0.1 in control (n = 17) and 5.2 ± 0.18 in BAPTA-treated (n = 12) cells. Because the products of hydrolysis of the acetoxymethyl ester of BAPTA can have nonspecific deleterious effects, we also evaluated the need for Ca\(^{2+}\) using thapsigargin. Prevention of the [Ca\(^{2+}\)] transient by depletion of the stores resulted in an even more pronounced inhibition of the acidification, which reached only 5.7 ± 0.2 (n = 12) (Fig. 8D). Jointly, these experiments indicate that FcRIIA receptor-mediated phagosomal maturation is partly dependent on increased [Ca\(^{2+}\)].

Functional Significance of Phagosomal Acidification—As illustrated in Fig. 2E, phagosomal acidification develops shortly after particle internalization. It has been speculated that the initial phase of acidification, likely induced by insertion of V-ATPases from early endosomes, is required for the successful fusion of phagosomes with lysosomes. This notion is based on findings using weak bases or ionophores to dissipate endosomal pH, with resulting inhibition of phagosome to lysosome fusion (39). However, the agents used to alter pH concomitantly affect the volume and ionic composition of acidic compartments. Such secondary effects complicate the interpretation of the role of pH gradients in fusion. To analyze the effect of dissipation of pH on the fusion of phagosomes with lysosomes, without simultaneously inducing swelling and/or ionic replacement, we inhibited the V-ATPase with concanamycin or bafilomycin prior to the induction of phagocytosis. As shown in Fig. 9A, the V-ATPase inhibitors had no significant effect on the ability of the transfected cells to internalize IgG-opsonized particles. More importantly, neither concanamycin nor bafilomycin altered the fusion of phagosomes with LAMP-1 or...
LAMP-2-containing vesicles (Fig. 9B). Parallel determinations ascertained that, when added prior to phagocytosis, the V-ATPase blockers completely prevented the development of phagosomal acidification (not shown). We conclude that acidification of the phagosomal vacuole is not required for effective phagosome to lysosome fusion.

Bacteriostatic Effect of FcRIIA Receptor-transfected Cells—The results above indicate that FcRIIA receptor-transfected cells can effectively recapitulate the processes of phagocytosis and phagosomal maturation. We therefore considered whether, like professional phagocytes, the transfected cells could limit the growth of bacteria. For these studies, CHO-2A cells were exposed to viable *Escherichia coli* for the indicated periods; the cells were then washed extensively, exposed to gentamicin for 1 h at 37 °C (to kill any remaining extracellular bacteria), and lysed with Triton X-100 (to release intracellular bacteria), and the lysates were plated on LB agar plates for bacterial quantitation. As shown in Fig. 10A, the transfectants were able to effectively internalize bacteria, but only after opsonization with an *E. coli*-specific antibody.

To assess the fate of the bacteria following internalization by CHO-2A cells, we compared their growth within the cells to that of bacteria that were released from phagosomes by treatment with detergent 1 h after ingestion, and suspended freely in culture medium. As illustrated in Fig. 10B, intracellular bacteria failed to replicate, and in fact their number decreased, although the extent of the reduction did not attain statistical significance. Over the same period of time, bacteria released from the cells shortly after ingestion proliferated actively, their number increasing by over 7 orders of magnitude. This indicates that the FcγRIIA receptor-transfected cells exerted an

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**FIG. 5. Co-localization of Tfn with internalized opsonized RBC.** A, distribution of internalized Tfn in CHO cells transfected transiently with the human Tfn receptor. B and C, cells transfected with human Tfn receptor were first allowed to internalize IgG-opsonized RBC for 15 min. Any adherent extracellular RBC that were not internalized were then lysed hypotonically. Next, the cells were incubated with labeled Tfn for 10 min, and the reaction was terminated by washing in ice-cold medium. The cells were visualized using fluorescence (B) and Nomarski (C) optics. Arrows point to RBC that colocalize with Tfn.

**FIG. 6. Co-localization of lysosomal markers with internalized particles in COS-2A cells.** A and B, localization of CD63 revealed by indirect immunofluorescence using Cy3-labeled antibodies. In A, the cells were otherwise untreated, whereas in B, the cells were allowed to internalize FITC-labeled zymosan particles for 1 h prior to fixation. The position of a particle that co-localizes with CD63 is indicated by the solid arrowhead, whereas unstained particles that were not internalized or had not fused with lysosomes are shown by open arrowheads. C, the location of the zymosan particles in B was revealed monitoring the fluorescence of FITC. Experiments like the one illustrated in A–C were also performed to reveal the distribution of LAMP-1 (D and E) and LAMP-2 (G and H), before (D and G) or after (E and H) phagocytosis of opsonized zymosan. The position of the respective zymosan particles is shown in F and I. Images are representative of at least three experiments of each kind.

LAMP-2-containing vesicles (Fig. 9B). Parallel determinations ascertained that, when added prior to phagocytosis, the V-ATPase blockers completely prevented the development of phagosomal acidification (not shown). We conclude that acidification of the phagosomal vacuole is not required for effective phagosome to lysosome fusion.

**FIG. 7. Kinetics of association of endosomal and lysosomal markers with phagosomes.** CHO-2A cells transfected with human Tfn receptor were preincubated with labeled Tfn for 1 h and then chased for 7 min, before exposure to IgG-opsonized RBC for 15 min. After lysis of adherent, noninternalized RBC, the CHO cells were incubated at 37 °C for the indicated periods of time (abscissa). The reaction was then stopped, and the fixed and permeabilized cells were used for immunostaining of LAMP-1. The fraction of phagosomes containing Tfn (squares) or LAMP-1 (circles) was assessed by combined Nomarski and epifluorescence microscopy. The data are means ± S.E. of three separate experiments, each with at least 50 phagosomes.
effective bacteriostatic effect and may have modest bactericidal capability as well.

The precise mechanism underlying bacteriostasis remains undefined, but phagosomal acidification is most likely to contribute to this effect, because the rate of replication of *E. coli* grown in culture broth decreases sharply below pH 5 (Fig. 10B, inset). As reported above, the phagosomal pH in FcγRIIA receptor-transfected cells can reach 4.84. We attempted to establish more directly whether phagosomal acidification was essential for the bacteriostatic action of the CHO-2A cells. However, the concentrations of bafilomycin and concanamycin required to inhibit phagosomal acidification directly and effectively killed *E. coli*. Moreover, prolonged exposure to these and other agents that dissipate endomembrane acidification proved toxic for CHO-2A cells.

**DISCUSSION**

The goal of this study was to determine whether nonmyeloid cells, rendered phagocytic by expression of human Fcγ receptors, possessed the cellular machinery that would allow functional maturation of the phagosome. Our data demonstrate that not only can such cells internalize opsonized particles rapidly and efficiently in a ligand-dependent manner but that the phagosome becomes progressively acidified by recruitment of V-ATPases from endomembrane compartments. Moreover, we observed that when actively dividing *E. coli* are internalized by this receptor-mediated pathway, they promptly cease to proliferate. Failure of the bacteria to grow within the transfected cells was not due to opsonization or to the phagocytic process itself, because *E. coli* released from phagosomes shortly after internalization proliferated actively in suspension. Instead, it is likely that the conditions generated within the phagosome upon fusion with lysosomes limit the growth of the bacteria. Lysosomal hydrolases and the low luminal pH probably contribute to this effect.

Fcγ receptors were shown earlier to modify the fate of intracellular vacuoles that encapsulate invading *Toxoplasma*. This microorganism generates a parasitophorous vacuole that neither fuses with lysosomes nor becomes acidic (24). When the vacuole contains active FcγRIIB1 or B2 receptors, fusion with lysosomes and acidification develop (24). Importantly, unlike FcγRIIA, the B1 and B2 isoforms are unable to trigger phagocytosis of IgG-coated inert particles, such as RBC (10, 40, 41). This implies that signals generated by interaction of Toxoplasma with other cellular components are required for FcγRIIB1 or B2-induced phagosome formation and maturation. In contrast, cross-linking of FcγRIIA suffices to induce phagosome formation (25) and to trigger its fusion with endosomes and lysosomes. This differential behavior has been attributed to the presence of an immunoreceptor tyrosine-based activation motif (ITAM) or ITAM-like sequence in the cytoplasmic tail of FcγRIIA receptors. Such an ITAM-like sequence is not present in human FcγRIIB1 or B2 (42–45). Concomitant phosphorylation of both ITAM tyrosine residues of FcγRIIA is thought to promote the attachment and subsequent activation of p72^tyk2^ via its dual SH2 domains (12, 46, 47). Clustering of p72^tyk2^ is in turn sufficient to promote phagocytosis (13), and failure to activate this kinase may account for the inability of FcγRIIB1 and B2 to initiate phagocytosis. It is likely that signals other than the activation of p72^tyk2^ must contribute to phagosomal maturation, inasmuch as FcγRIIB1 and B2 facilitate lysosomal fusion and acidification, at least in the case of Toxoplasma-induced vacuoles (24).

As in the case of macrophages, phagosomes formed by IgG-opsonized particles in FcγRIIA-transfected cells fused sequentially with early endosomes and subsequently with late endosomes and lysosomes (Fig. 7). The resulting phagolysosomes acidified to levels comparable to those reported in macrophages (48, 49). The acidification was reduced but not eliminated by chelation of intracellular calcium or by depletion of intracellular calcium stores. In this regard, the transfected cells resemble neutrophils, in which prevention of calcium transients inhibits phago-lysosomal fusion (19), but they differ from macrophages, in which no effect was noted (37). It remains unclear whether these two closely related cell types utilize fundamentally different fusion mechanisms, or whether methodological differences account for the apparent discrepancies. It is noteworthy, in this regard, that fusion systems heretofore thought to be calcium-independent are being found to require the divalent
Fig. 10. Ability of FcγRIIA receptor-expressing cells to internalize and kill opsonized E. coli. A, CHO-2A cells were incubated with either opsonized or unopsonized bacteria (1 × 10^9 per well of a six-well plate). After the indicated incubation, the cells were washed six times with sterile PBS and allowed to internalize any adherent bacteria for 1 h. After killing adherent extracellular bacteria using 100 μg/ml gentamicin, the CHO-2A cells were lysed with 0.2% Triton X-100, and serial dilutions of the resulting cell lysates were spread onto LB plates. The colonies resulting from an overnight incubation at 37 °C were counted to quantify internalized bacteria. Results are an average of two experiments and are expressed as a percentage of the largest number of internalized bacteria. For each well of a six-well plate, the number of colonies ranged from 2 × 10^3 to 10 × 10^3. B, growth of bacteria within phagosomes of CHO-2A cells. Bacterial uptake was allowed to occur for 1 h, followed by washing and killing of extracellular bacteria as in A. The cells were then lysed with 0.2% Triton X-100, and aliquots of the lysate were used to inoculate LB medium, which was cultured for the indicated periods (squares) or overnight (triangles) (n = 3). Alternatively, the CHO-2A cells were incubated in Dulbecco’s modified Eagle’s medium + 10% serum and 5 mg/ml of gentamicin for a further 18 h after phagocytosis, before lysis with Triton X-100 (triangles) (n = 7). Serial dilutions of the cell lysate or liquid culture were spread onto LB plates, and the colonies resulting from an overnight incubation at 37 °C were counted. Results are expressed relative to the number of bacteria internalized by the cells after 1 h of phagocytosis (time 0 in B). Data are means ± S.E. Where absent, error bars are smaller than the symbol. Inset, growth rate of E. coli in media of varying pH. The bacteria were grown for 6 h in LB broth titrated to the indicated pH. Results are means ± S.E. of three experiments. Where absent, error bars are smaller than symbol.

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