Involvement of p21\textsuperscript{\textit{racA}}, Phosphoinositide 3-Kinase, and Vacuolar ATPase in Phagocytosis of Bacteria and Erythrocytes by 
\textit{Entamoeba histolytica}: Suggestive Evidence for Coincidental Evolution of Amebic Invasiveness

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Trophozoites of \textit{Entamoeba histolytica}, the protozoan parasite that causes amebic dysentery, phagocytose bacteria in the colonic lumen and erythrocytes (RBC) in host tissues. Because tissue invasion is an evolutionary dead end, it is likely that amebic pathogenicity is coincidentally selected, i.e., the same methods used to kill bacteria in the colonic lumen are used by parasites to damage host cells and cause disease. In support of this idea, the amebic lectin and pore-forming peptide are involved in binding and killing, respectively, bacteria and host epithelial cells. Here amebic phagocytosis of bacteria, RBC, and mucin-coated beads was disrupted by overexpression of \textit{E. histolytica} p21\textsuperscript{\textit{racA-V12}}, a ras-family protein involved in selection of sites of actin polymerization, which had been mutated to eliminate its GTPase activity. p21\textsuperscript{\textit{racA-V12}} transformants were also defective in capping and cytokinesis, while pinocytosis of fluorescent dextrans was not affected. Wortmannin, a fungal inhibitor of phosphoinositide 3-kinase, markedly inhibited phagocytosis of bacteria, RBC, and mucin-coated beads by wild-type amebae. In contrast to p21\textsuperscript{\textit{racA-V12}} overexpression, wortmannin abolished amebic pinocytosis of dextrans but had no inhibitory effects on capping. Inhibition of amebic vacuolar acidification by bafilomycin also decreased bacterial and RBC uptake. These results, which demonstrate similarities between mechanisms of phagocytosis of bacteria and RBC by amebae and macrophages, support the idea of coincidental selection of amebic genes encoding proteins that mediate destruction of host cells.

\textit{Entamoeba histolytica} is an amitochondriate protozoan parasite, which causes dysentery and liver abscess in developing countries, where its fecal-oral spread cannot be prevented (34, 40). \textit{Entamoeba dispar} is a closely related enteric protozoan parasite, which does not cause dysentery or liver abscess (15). Trophozoites (ameboid forms) of \textit{E. histolytica} and \textit{E. dispar} grow under anaerobic conditions in the lumen of the distal colon, where they consume bacteria as their nutrient source (36, 44). In the colonic lumen, trophozoites transform to cysts, which have four nuclei and a chitin wall. Cysts are the infectious stage of the parasite, because they are resistant to attack by stomach acids, duodenal proteases, and bile (40). Tissue invasion by \textit{E. histolytica} is an evolutionary dead end because trophozoites do not form cysts in tissues, patients with amebic dysentery shed fewer infectious cysts and more noninfectious trophozoites, and liver abscesses are potentially fatal to the host (6, 8, 34).

In the absence of selection, it is not obvious how \textit{E. histolytica} genes involved in tissue invasion are retained. Here we suggest that these genes, which encode amebic proteins that damage host cells, are maintained by coincidental selection, i.e., these same proteins are involved in phagocytosis of bacteria within the colonic lumen, where amebae do not cause disease (19, 29, 35, 43). In support of the idea of coincidental selection, phagocytosis-deficient parasites selected with bromodeoxyuridine-labeled bacteria are less pathogenic than wild-type parasites (46), addition of bacteria to axenic parasites causes them to be more virulent (36), lectins mediate amebic phagocytosis of bacteria and epithelial cells (32, 36, 42), and amebae make pore-forming peptides (PFP; also known as amebapores), which are lytic to bacteria and epithelial cells (27, 28). Similarly, NK lysins, which are mammalian homologs of amebic PFP, are lymphocyte proteins involved in bacterial killing and tumor cell lysis (3). Other infectious examples of coincidental selection include \textit{Escherichia coli} urinary tract infections, which are mediated by pili that also attach bacteria to gastrointestinal epithelial cells, and cerebral malaria, which involves knobs that also sequester parasites away from the spleen (29, 33).

A prediction of the idea of coincidental selection amebic tissue invasion is that proteins involved in host cell lysis will also be involved in bacterial killing. In the case of the amebic lectin and PFP, these experiments have already been performed (27, 28, 32, 42). Our goal was to study a number of other amebic proteins which may be involved in host cell and bacterial killing. Three amebic proteins (p21\textsuperscript{\textit{rac}}, phosphoinositide 3-kinase [PI 3-kinase], and vacuolar proton-transporting ATPase [V-ATPase]) were tested because they are involved in killing of bacteria and senescent erythrocytes (RBC) by macrophages (4, 12, 18, 22, 31, 37). p21\textsuperscript{\textit{rac}} proteins, members of the \textit{ras} family of signal-transducing proteins, are low-molecular-weight GTP-binding proteins, important for site selection of actin polymerization (1, 22, 38, 45). Amebae have at least seven different \textit{ras} genes, one of which encodes p21\textsuperscript{\textit{rac}}, which shows \textgreater;70\% positional identities with p21\textsuperscript{\textit{rac}} of \textit{Dictyostelium discoideum}, humans, \textit{Drosophila melanogaster}, and \textit{Caenorhabditis elegans} (10, 11, 21, 31, 49).

PI 3-kinases are a family of enzymes with 100-kDa catalytic subunits and an 85-kDa regulatory subunit, which has SH2, SH3, and BCR domains (50). PI 3-kinases, which phosphorylate phosphoinositides, are involved in signal transduction in...
response to insulin, nerve growth factor, histamine, and some cytokines. PI 3-kinases are irreversibly inhibited by the cell-permeable fungal protein wortmannin, which covalently modifies Lys-802 in the catalytic subunit (54). Wortmannin inhibits macrocytophosis, Fcγ receptor signaling, and phagocytosis by macrophages (4, 37).

Phagolysosomal acidification, which is inhibited by the weak base monensin or ammonium chloride, is necessary for contact-mediated cytosis of host epithelial cells by amebae (41, 48). Bafilomycin A, a specific inhibitor of V-ATPases, inhibits acidification of pinocytotic vacuoles (7, 30). Amebic genes encoding the catalytic peptide (ATPase) and proteolipid (transmembrane domain) of the V-ATPase have been cloned previously (14, 47, 55). Vacular acidification is also essential for bacterial killing by macrophages (18).

Here cultured amebae were transformed with an E. histolytica racA gene encoding a p21<sup>racA</sup>-mutant protein with a Gly-12-Val mutation, which eliminates its GTPase activity and causes this regulatory protein to remain constantly in the active state. The ability of p21<sub>racA-V12</sub>-transformed amebae to phagocytose bacteri, RBC, and mucin-coated beads was determined (17). Transformed amebae were also incubated with fluorescein isothiocyanate (FITC)-conjugated dextran and FITC-concanavalin A (ConA) to determine the effect of p21<sup>racA-V12</sup> overexpression on pinocytosis and capping, respectively (16, 30). The effects of wortmannin, bafilomycin, monensin, and ammonium chloride on phagocytosis of bacteria and RBC by wild-type amebae were also determined.

The last tests performed were amebic cysteine proteases (encoded by at least five genes), which have been implicated in epithelial cell destruction, degradation of the extracellular matrix, and abscess formation (9, 24, 39, 51). The cysteine protease inhibitor E-64, at concentrations that inhibited abscess formation, as well as three other cysteine protease inhibitors, was assayed for its ability to inhibit amebic phagocytosis of bacteria and RBC.

**MATERIALS AND METHODS**

**Construction of the pJST4-RacA-V12 vector and transformation of E. histolytica.** The E. histolytica racA gene was modified in two ways before cloning into the pJST4 vector using synthetic oligonucleotides and PCR (Fig. 1) (17). First, 27 bp encoding the hemagglutinin antigen (HA) of influenza virus (YPYDVPDYA) was added to the 5′ end of the racA gene (the motif terminus of p21<sup>racA</sup>) (53). Second, E. histolytica p21<sup>racA</sup> was aligned with human p21<sup>racA</sup> to identify the Gly residue, which, when mutated to Val, disrupts the GTPase activity of the proteins (45). The twice-mutagenized E. histolytica racA gene was modified in two ways before cloning into pJST4 to make the pJST4-RacA-V12 vector, which was sequenced to confirm the presence of the intended modifications. The pJST4-RacA-V12 vector was electroporated into E. histolytica HM-1 amebae and selected with G418 to a final concentration of 50 μg/ml (22).

Expression of p21<sub>racA-V12</sub> by transformed parasites was confirmed in two ways. First, total RNA of transformed and nontransformed parasites (control) was purified with an RNA Easy kit (Qiagen) and treated with RNase-free DNase (Pharmacia) to remove contaminating DNA. Reverse transcriptase PCR (RT-PCR) was performed with primers to the HA epitope and to the 3′ end of the racA gene. Positive controls included primers to amebic actin genes, while negative controls omitted RT. Second, total proteins of amebae were lysed in sodium dodecyl sulfate and β-mercaptoethanol, electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels containing 15% acrylamide, and transferred to polyvinylidene difluoride membranes. Membranes were incubated with a mouse monoclonal antibody against the HA epitope (Boehringer), and bound mouse antibodies were detected with a chemiluminescence kit (Amerham) (55).

Light and scanning electron microscopy of cultured parasites. Nontransformed amebae and p21<sub>racA-V12</sub>-transformed parasites were observed in 50-ml flasks with an inverted-phase microscope to determine whether there were dysmorphic amebae among the latter. Differences in the rate of cytokinesis were estimated by measuring the time it took for 20 nontransformed amebae and 20 p21<sub>racA-V12</sub> transformants to complete binary fission.

To test the effects of wild-type parasites with wortmannin, ammonium chloride, monensin, and E-64, 100 μM wortmannin or 30 mM ammonium chloride, and the same concentration of drug was maintained after addition of bacteria, RBC, mucin-coated beads, FITC-dextran, or FITC-ConA (4, 37, 54). To test the effect of inhibition of vacuolar acidification, amebae were preincubated with 100 μM monensin, or 30 mM ammonium chloride, and the same concentration of each drug was maintained after addition of bacteria or RBC (7, 18, 30). To test the effect of inhibition of cysteine proteases, amebae were preincubated with 100 μM E-64, 10-μg/ml Z-Leu-Lys-O-Tyr-CH<sub>3</sub>, 100-μg/ml phenylmethylsulfonyl fluoride, or 5-μg/ml leupeptin, and the same concentration of drug was maintained after addition of bacteria (9, 24, 39).

**RESULTS**

Morphological comparison of nontransformed amebae and p21<sub>racA-V12</sub> transformants. Overexpression of p21<sub>racA-V12</sub> by transformed parasites, selected with G418, was confirmed by RT-PCR with primers to the HA epitope and to the 3′ end of the racA gene and by Western blotting with an anti-HA epitope monoclonal antibody (Fig. 1). Control RT-PCR and Western blotting were negative with nontransformed parasites. The pJST4-RacA-V12 plasmid was recovered intact from transformants.

**REFERENCES**

Phagocytes as shown by scanning microscopy (22, 38, 45). Phalloidin staining of filamentous actin demonstrated a second defect in these mutant parasites. Prominent actin stress fibers and cup-like accumulations of actin under the plasma membrane were much more frequent in p21^racA-V12 transformants than in nontransformed parasites (Fig. 2). In nontransformed amebae, prominent actin stress fibers are rare, while cup-like accumulations of actin are associated with phagocytosis of RBC (5, 13, 16).

**Interactions of p21^{racA-V12} transformants with their environment.** Overexpression of p21^{racA-V12} had profound effects on rates of phagocytosis of bacteria, RBC, and mucin-coated beads and inhibited amebic capping but had no effect on pinocytosis of dextran or lysis of phagocytosed bacteria and RBC (see Fig. 3 to 7).

**Phagocytosis of bacteria.** In the colonic lumen, amebae consume bacteria by phagocytosis (36). *E. coli* cells expressing recombinant GFP under an IPTG-inducible promoter were used to study amebic phagocytosis because these bacteria were very bright and easy to see when phagocytosed by amebae (Fig. 3) (51). More than 40% of nontransformed amebae phagocytosed >10 bacteria/ameba within 10 min, while 25% of these amebae phagocytosed <6 bacteria. Heterogeneity in the number of bacteria (as well as RBC and beads [see below]) consumed by nontransformed amebae was likely secondary to the use of unsynchronized parasites in these studies. In contrast, only 9% of p21^{racA-V12} transformants phagocytosed >10 bacteria, while 52% of p21^{racA-V12} transformants phagocytosed <6 bacteria. These results suggest that overexpression of p21^{racA-V12} disrupts amebic phagocytosis of bacteria, as has been shown elsewhere for macrophages (12).

Killing of phagocytosed bacteria, judged by release of GFP into the vacuole, however, appeared similar for nontransformed amebae and p21^{racA-V12} transformants (Fig. 3). Both groups of parasites started killing bacteria after ~30 min of intake, and killing was nearly complete for both groups after 90 min. These killing times are somewhat shorter than those previously reported for amebae but are not dissimilar to those reported for macrophages elsewhere (4, 20).

**Phagocytosis of RBC.** p21^{racA-V12} transformants were also defective in the uptake of human RBC versus nontransformed amebae (Fig. 4). Most of the bound RBC were taken into phagocytic vacuoles of nontransformed amebae within 10 min at 37°C. In contrast, most of the bound RBC remained on the surface of p21^{racA-V12} transformants, often in large aggregates. Sixty percent of nontransformed amebae phagocytosed >10 RBC per parasite, while 25% took up <6 RBC. In contrast, 15% of p21^{racA-V12} transformants phagocytosed >10 RBC, while 65% phagocytosed <6 RBC. Lysis of phagocytosed RBC, detected by spilling of hemoglobin pigment into the vacuole, appeared to be unchanged for p21^{racA-V12} transformants (data not shown). Lysis of RBC and bacteria by the transformants suggests that fusion of phagosomes with lysosomes was not impaired by overexpression of hyperactive p21^{racA}.
Phagocytosis of beads. Parasites took up few beads that were uncoated, negatively charged, or positively charged (data not shown). In contrast, amebae rapidly phagocytosed mucin-coated beads, demonstrating that bead uptake is specific and is likely accomplished by means of lectins on the parasite surface (Fig. 5) (32, 42). Mucin beads were very bright and of uniform size, did not quench with prolonged UV exposure, were not degraded by the parasites, and so were easy to count with the fluorescence microscope. Nontransformed parasites consumed $16 \pm 9$ beads/parasite (average and standard deviation, respectively), with 67% taking up $>11$ beads. In contrast, $p21^{noc4-V12}$ transformants consumed $10 \pm 8$ beads/parasite ($P < 0.001$ versus nontransformed amebae), with only 37% taking up $>11$ beads.

Pinocytosis. Amebae pinocytose as much as 30% of their volume per hour (30). Here nontransformed amebae and $p21^{noc4-V12}$ transformants took up FITC-dextran into small vacuoles (as shown in Fig. 1 of reference 47) at nearly the same rates when measured by FACS (Fig. 6). This result suggests that amebic pinocytosis was not disrupted by overexpression of $p21^{noc4-V12}$.

Capping. Capping of bound antibodies and their release by shedding, which is mediated by actin and myosin II, have been suggested as a mechanism by which amebae avoid the host immune response (2, 16, 20). Ninety-six percent of all nontransformed amebae form a single cap on their surface when incubated with the multivalent ligand FITC-ConA, while 4% had two caps (Fig. 7). In contrast, only 71% of $p21^{noc4-V12}$ transformants formed a single cap, 14% of transformed amebae had two caps per cell, 5% had three caps per cell, and 10% had no caps. Similar capping results were seen when a mutated $p21^{nocG}$ was overexpressed (21).

Wortmannin inhibited amebic pinocytosis and phagocytosis of bacteria, RBC, and mucin-coated beads but had no effect on capping. Wortmannin, an inhibitor of PI 3-kinase, completely abolished uptake of bacteria and RBC by amebae (data not shown). Only 38% of wortmannin-treated amebae took up $>10$ beads, a result similar to that for $p21^{noc4}$ transformants (Fig. 5). Wortmannin also completely inhibited pinocytosis of FITC-dextran (Fig. 6). Wortmannin inhibits pinocytosis of high-molecular-weight dextrans and phagocytosis by macrophages (4). Wortmannin had no effect on amebic capping, as 95% of parasites treated with wortmannin formed a single, normal-looking cap with FITC-ConA, while 4% had two caps, and 1% had no cap. These results are different from those for...
overexpression of \( p21^{racA-V12} \), in which capping was inhibited in many cells while pinocytosis was not affected.

**Raising vacuolar pH inhibited phagocytosis of bacteria and RBC.** Inhibition of vacuolar acidification partially inhibits amebic pinocytosis and blocks amebic lysis of epithelial cells in vitro (30, 41). Vacuolar pH was raised in three ways: by addition of two weak bases (monensin and ammonium chloride) and by inhibition of the V-ATPase by bafilomycin (7). In all three cases, bacterial uptake and killing were greatly reduced (Fig. 3). Zero percent of ameba treated with bafilomycin, 3% of those treated with monensin, and 3% of those treated with ammonium chloride phagocytosed >10 bacteria/parasite. Similarly, 98% of ameba treated with bafilomycin, 48% of those treated with ammonium chloride phagocytosed >10 bacteria/parasite. Similarly, 98% of ameba treated with bafilomycin, 48% of those treated with monensin, and 92% of those treated with ammonium chloride phagocytosed <6 bacteria. Bafilomycin also greatly inhibited uptake of RBC (Fig. 4). Three percent of bafilomycin-treated parasites phagocytosed >10 RBC per parasite, while 81% took up <6 RBC. These results, which are consistent with the coincidental selection of amebic lysis of host cells, are likely secondary to inhibition of amebic lysozyme and PFP, each of which has an acid pH optimum (23, 27, 28, 48). Inhibition of vacuolar acidification also blocks killing of bacteria by macrophages (18).

**Inhibition of amebic cysteine proteinase and other proteases had no effect on phagocytosis.** Inhibition of amebic cysteine proteinase by E-64 blocks amebae from forming liver abscesses in vivo (51). In contrast, E-64 and three other cysteine proteinase inhibitors (\( Z-L-Leu-L-Tyr-CHN_2 \), phenylmethylsulfonyl fluoride, and leupeptin) had no effect on amebic phagocytosis of bacteria and RBC. These results suggest that the cysteine proteinase is not necessary for lysis of bacteria and RBC. Whether protease inhibitors block degradation of proteins released from lysed bacteria and RBC could not be determined.

**DISCUSSION**

Comparison of amebic phagocytosis with that of macrophages. Multiple methods developed to examine macrophage function were used for the first time to study amebic phagocytosis: (i) overexpression of \( p21^{racA-V12} \), which lacks GTPase activity and so is fixed in the active state, (ii) incubation of parasites with fluorescent beads and GFP-labeled bacteria, and (iii) addition of wortmannin and bafilomycin to phagocytosing parasites (4, 7, 17, 22, 25, 37, 38, 45, 52, 54). Although amebae diverged from the main eukaryotic lineage about a billion years ago, there are numerous similarities between amebic killing of bacteria and that by human leukocytes. Like macrophages, amebae appear to need intact \( p21^{racA} \), PI 3-kinase, and V-ATPase for phagocytosis of bacteria and RBC, and amebae may be activated to kill host epithelial cells by incubation with bacteria (4, 12, 18, 25, 37, 41). Like lymphocytes, amebae have...
the failure of tions in the colonic lumen and in tissue abscesses and ameboid pathogenesis include unique fermentation enzymes that make Other likely examples of coincidental selection for amebic or degradation of cyst wall by excysting parasites (26, 48).

Overexpression of p21rac in cultured amebae has numerous effects that might be expected: inhibition of capping and decreased phagocytosis of mucin-coated beads, RBC, and bacteria (2, 13, 18, 21). Overexpression of p21rac also had some less predictable effects: decreased rates of cytokinesis and formation of actin cups in the absence of external stimuli (5, 20). Although these overexpression experiments cannot demonstrate the normal function of the amebic p21rac, they suggest possible functions in which this or other amebic p21rac proteins might be involved. These experiments also suggest functions in which p21rac might not be involved (e.g., pinocytosis and fusion of phagosomes and lysosomes) (30, 48).

Coincidental selection. The idea of coincidental selection is important to the extent that it explains how amebic pathogenicity is maintained without obvious survival advantage to the parasite (19, 29, 38). Bacterial lysis and epithelial cell lysis have at least five common mechanisms: lectin binding and amebic pore lysis (shown in references 3, 27, 28, 32, and 42) and involvement of p21rac, PI 3-kinase, and V-ATPase (shown here). Remarkably, p21rac overexpression and PI 3-kinase inhibition have similar effects on phagocytosis but opposite effects on pinocytosis and capping. Although the experiments with the cysteine proteinase inhibitors failed to support the idea of coincidental selection, it is not difficult to imagine other functions for the amebic cysteine proteinases besides bacterial killing (e.g., autodegradation of parasite proteins in lysosomes or degradation of cyst wall by excysting parasites) (26, 48). Other likely examples of coincidental selection for amebic pathogenesis include unique fermentation enzymes that make it possible for parasites to reproduce under anaerobic conditions in the colonic lumen and in tissue abscesses and ameboid motility that allows parasites to capture bacteria and penetrate into host tissues (2, 13, 44).

What is not explained by the idea of coincidental selection is the failure of E. dispar parasites, which also consume bacteria in the colonic lumen, to cause tissue disease and the inability of amebae to reproduce and form cysts in the colonic lumen of other mammalian hosts (15, 40, 43). Although there are quantitative and qualitative differences in lectins, PFP, and cysteine proteinases of E. histolytica and E. dispar, it is possible that amebic tissue invasion is determined by factors unrelated to bacterial killing (9, 24, 28, 32, 39). For example, genetic studies of Salmonella bacteria have shown that a handful of virulence genes are involved in host recognition and invasion, while the vast majority of bacterial virulence genes are involved in transcriptional control, nutrient biosynthesis, and peptide resistance (19). When methods for gene knock-out and transposon-mediated mutagenesis are developed for amebae, numerous new genes of the parasite involved in tissue invasion will likely be identified and the idea of coincidental selection may be tested further.

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