Dictyostelium discoideum Mutants with Conditional Defects in Phagocytosis

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Abstract. We have isolated and characterized *Dictyostelium discoideum* mutants with conditional defects in phagocytosis. Under suspension conditions, the mutants exhibited dramatic reductions in the uptake of bacteria and polystyrene latex beads. The initial binding of these ligands was unaffected, however, indicating that the defect was not in a plasma membrane receptor. Because of the phagocytosis defect, the mutants were unable to grow when cultured in suspensions of heat-killed bacteria. The mutants exhibited normal capacities for fluid phase endocytosis and grew as rapidly as parental (AX4) cells in axenic medium. Both the defects in phagocytosis and growth on bacteria were corrected when the mutant *Dictyostelium* cells were cultured on solid substrates. Reversion and genetic complementation analysis suggested that the mutant phenotypes were caused by single gene defects. While the precise site of action of the mutations was not established, the mutations are likely to affect an early signaling event because the binding of bacteria to mutant cells in suspension was unable to trigger the localized polymerization of actin filaments required for ingestion; other aspects of actin function appeared normal. This class of conditional phagocytosis mutant should prove to be useful for the expression cloning of the affected gene(s).

THE ability of eukaryotic cells to internalize large particles by phagocytosis was first described by Metschnikoff more than 100 years ago (Metschnikoff, 1887). It has also long been appreciated that phagocytosis of microorganisms and damaged or senescent cells plays a critical role in host defense, tissue morphogenesis, and infection by intracellular pathogens (Falkow et al., 1992; Silverstein et al., 1989). Nevertheless, despite this long history, little is known about the mechanisms or regulation of phagocytosis in either vertebrate or invertebrate cells.

Phagocytosis involves the uptake of particles or cells >1.0 μm in diameter. It is initiated only by the binding of particles to a specific subset of plasma membrane receptors capable of triggering the series of events leading to ingestion (Silverstein et al., 1977, 1989). One critical event is the localized polymerization of actin filaments directly beneath the site of particle attachment. Together with myosin I, paxillin, and talin, the actin filaments provide the motile force that drives, by an as yet unspecified mechanism, pseudopod outgrowth that results in the internalization of the target particle (Fukui et al., 1989; Greenberg et al., 1990, 1994; Griffin et al., 1976; Jung and Hammer, 1990; Stossel, 1989). Phagocytosis can either be an ordered process, involving a progressive migration of pseudopodia around the circumference of the particle being internalized (Griffin et al., 1975, 1976), or a disordered process in which particles are ingested as a result of having induced localized membrane ruffling (Francis et al., 1993). In either event, it is blocked by agents such as cytochalasin that interfere with the actin-containing cytoskeleton (Axline and Reaven, 1974; Francis et al., 1993; Malawista et al., 1971). Dependence on the actin cytoskeleton is one feature that distinguishes phagocytosis from pinocytosis (or receptor-mediated endocytosis), which is more commonly dependent on clathrin. Like material internalized by pinocytosis, ingested particles are degraded intracellularly after the fusion of phagocytic vacuoles with late endosomes and lysosomes (Rabinowitz et al., 1992; Silverstein et al., 1977; Ukkonen et al., 1986).

Although virtually all cell types are capable of pinocytosis and receptor-mediated endocytosis, phagocytosis is most often associated with specialized cells of the immune system, such as monocytes and granulocytes. That is not to say that "nonprofessional" cells (e.g., fibroblasts) are incapable of phagocytosis, but only that their capacity is relatively limited (Joiner et al., 1990). This reflects, at least in part, differences in the receptors and signaling molecules expressed by phagocytic vs nonphagocytic cells. For example, macrophages abundantly express those receptors capable of triggering phagocytosis (Fc receptors, complement receptors, and mannose receptors), but these same receptors are generally not

present on nonphagocytic cells (Silverstein et al., 1977). While features or sequence motifs common to such receptors have not yet been identified, they all must participate directly or indirectly in signal transduction. One well-studied example is the macrophage IgG Fc receptor. Fc receptor–mediated phagocytosis is preceded by a transient increase in cytosolic free Ca^{++}, although the Ca^{++} flux is not actually required for particle uptake (Di Virgilio et al., 1988; Greenberg et al., 1991; Hishikawa et al., 1991; Lew et al., 1985; McNeil et al., 1986). More importantly, phagocytosis is associated with a marked increase in tyrosine phosphorylation at the site of phagocytic vacuole formation; drugs that inhibit phosphorylation reduce Fc receptor–mediated phagocytosis in macrophages (Greenberg et al., 1993).

Phagocytosis then involves a complex series of events involving signal transduction by specific receptors, a patterned reorganization of the actin-containing cytoskeleton, and transmission of the motile force generated by this reorganization to allow for plasma membrane envelopment of the phagocytic stimulus. Unfortunately, none of these events are understood at the molecular level. Because it has proven difficult to obtain this information using conventional techniques, we have begun to explore a genetic approach by studying phagocytosis in *Dictyostelium discoideum*, a free living soil ameba that feeds by ingesting bacteria.

Phagocytosis in *Dictyostelium* is strikingly similar to phagocytosis in mammalian leukocytes. *Dictyostelium* cells exhibit the same type of cytoskeletal reorganization as is found in neutrophils, involving the polarized reorganization of actin filaments and myosin I around the conserved actin binding membrane protein ponticulin (Chia et al., 1993; Fukui et al., 1989; Shariff and Luna, 1990; Stossel, 1989; Wuestehube et al., 1989). Unlike the phagocytes of higher organisms, however, *Dictyostelium* is amenable to molecular genetic manipulation (De Lozanne and Spudich, 1987; Jung and Hammer, 1990; Knecht and Loomis, 1987; Witke et al., 1992). Like yeast, *Dictyostelium* has a small haploid genome, and it is suitable for mutant selection. Since axenic strains of *Dictyostelium* can also grow in liquid medium where they appear to derive their nutrients by pinocytosis, we reasoned that it should be possible to isolate cells with conditional lethal mutations in phagocytosis that are unable to grow on bacteria but multiply normally in liquid culture.

Previous attempts to isolate phagocytosis-defective *Dictyostelium* mutants, either by screening for motility defects (Clarke, 1978; Kayman et al., 1982) or by screening for the inability to bind tungsten beads (Vogel et al., 1980), have been met with limited success. Thus far, the only confirmed cell lines unable to mediate phagocytosis have defects in particle binding as opposed to uptake (Vogel et al., 1980). By directly screening for cells unable to ingest fluorescent bacteria, we have now been able to isolate *Dictyostelium* mutants with severe, conditional-lethal defects in phagocytosis.

Materials and Methods

Strains and Growth Conditions

All mutants were derived from the axenic strain AX4 (a gift from David Knecht, University of Connecticut, Storrs, CT). Cells were grown in HL5 medium at 21°C in either 10-cm tissue culture dishes or Erlenmeyer flasks shaking at 150 rpm in a refrigerated air incubator (Sussman, 1987). Cells

were also cultured in suspensions of *Klebsiella aerogenes* that had been freeze thawed, autoclaved, and resuspended to 50 mg/ml in 20 mM phosphate buffer, pH 6.3. Spores were obtained after growth of cells on sm-1 plates, and subsequently stored by drying onto silica chips or freezing at −20°C in 20 mM phosphate buffer, pH 6.3, with 15% glycerol (Sussman, 1987). Cell number was monitored with a hemacytometer. Cells were collected by centrifugation at 100 g for 5 min.

Fluorescent Labeling of Bacteria

To measure phagocytosis, we monitored the uptake of different FITC-labeled bacterial strains using a modification of the method of Vogel (1980). To prepare fluorescently labeled bacteria, the desired bacterial strain was grown at 37°C in Luria broth to $OD_{600} = 2$. Bacteria were pelleted and resuspended in 10 ml of 50 mM phosphate buffer, pH 9.2, containing 0.1 mg/ml fluorescein isothiocyanate (Sigma Immunochemicals, St. Louis, MO). The mixture was shaken at 150 rpm for 1 h at 21°C and was then washed by repeated centrifugation in 20 mM phosphate buffer, pH 6.3, until all unreacted fluorescein was removed. The bacteria were then resuspended to 2.5×10^{10} bacteria/ml in phosphate buffer, pH 6.3.

Quantitation of Bacterial Uptake

Dictyostelium growing exponentially in HL5 were resuspended to a density of 4×10^6 cells/ml and mixed with bacteria at a concentration of 5×10^9 bacteria/ml in 2 ml of the specified media. Cells were agitated at 150 rpm in a 25-ml Erlenmeyer flask on a rotary shaker at 21°C. Uptake was stopped by diluting 1 ml of the suspension in 10 ml of cold phosphate buffer. Samples were centrifuged for 5 min at 100 g to pellet the *Dictyostelium* but not uningested bacteria. The supernatant containing the noningested bacteria was removed, and the pellet was resuspended in 10 ml cold phosphate buffer. This procedure was repeated two additional times and effectively separated the *Dictyostelium* from the majority of the noninternalized bacteria. After the third centrifugation, cells were resuspended in 3 ml of 50 mM phosphate buffer, pH 9.2, counted, and lysed by the addition of Triton X-100 to a final concentration of 0.2%. The fluorescence was measured with a spectrofluorometer (LS-5; Perkin-Elmer Corp., Norwalk, CT) using excitation and emission wavelengths of 490 and 520 nm, respectively. To determine the total number of bacteria ingested, this value was compared with a standard curve generated by lysing defined numbers of bacteria in a 9.2-pH phosphate buffer containing 1% SDS. All measurements were performed in duplicate.

Phagocytosis of Latex Beads

Phagocytosis of polystyrene latex beads was assayed in essentially the same manner as bacteria. Briefly, 1-μm latex beads (5×10^9 beads/ml; Du Pont, Wilmington, DE) were incubated with *Dictyostelium*, and noningested beads were removed using the bacterial wash procedure described above. After counting, cells were lysed, and the OD_{600} of the resulting suspension was measured. This value was compared to a standard curve to determine the total number of cell-associated latex beads.

Phagocytosis of Bacteria on Filters

5×10^9 bacteria in 100 μl phosphate buffer were rapidly added to a pellet of 1×10^7 cells and immediately deposited onto polycarbonate filters (0.4-μm pore size; Costar Corp., Cambridge, MA). After a 30-min incubation in a humidified 21°C chamber, cells were resuspended by pipetting 1 ml of cold phosphate buffer over the filters. The cells were added to 9 ml of additional buffer and centrifuged for 5 min at 100 g to separate the *Dictyostelium* from the uningested bacteria. Cells were washed two more times, and the total number of internalized particles was determined as previously described.

Bacterial Strains

The following strains of bacteria were used for uptake, none of which are mucoid (the structures of the core or O-polysaccharides are also listed); *Salmonella minnesota* Re595 (lipid A-KDO-KDO), *Salmonella typhimurium* Rc (*Lipid A-KDO-KDO-Hep(PO4)-Hep-Glc*), S. *typhimurium* Ra (Lipid A-KDO-KDO-Hep(PO4)-Hep-Glc-Gal(Gal)-Glc-GlcNAc), *Escherichia coli* K12 (Lipid A-KDO-KDO-Hep(PO4)-Hep-Glc-Glc(Gal)-Glc-GlcNAc), *Salmonella adelaide* (Lipid A-Core-[Glc-Gal-Rha-Col]$_n$), S.

typhimurium 5771 (Lipid A-Core-[Man(Abe)-Rha-Gal]_n), *Salmonella en-teritidis 4340* (Lipid A-Core-[Man-(Tyv)-Rha-Gal]_n), *Salmonella mon-tevideo 5770* (Lipid A-Core[Man-Man(Glc)-Man-Man-GlcNAc]_n) *S. min-nesota 218* (Lipid A-Core-[GalNAc(GlcNAc)-GalNAc-Gal(Gal)]_n) Core = KDO-KDO-Hep(PO4)-Hep-Glc-Gal(Gal)-Glc-GlcNAc. Abbreviations: Abe, abequose; Man, D-mannose; Rha, L-rhamnose; Gal, D-galactose; Gal-NAc, N-acetyl-D-galactosamine; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; Hep, L-glycero-D-mannoheptose; KDO, 3-deoxy-D-manno-octulosonic acid; (Schweinle et al., 1989; Valtonen et al., 1975; Westphal et al., 1983).

Fluid Phase Endocytosis Assay

2 mg/ml FITC-dextran (Sigma Immunochemicals) was incubated for 45 min with cells suspended in HL5 at a density of 4×10^6 cells/ml at 21°C. Cells were diluted into 10 ml cold phosphate buffer and were washed three times by centrifuging at 100 g for 5 min. The cells were resuspended in 3 ml of 50 mM phosphate buffer, pH 9.2, counted, and lysed in 0.2% Triton X-100. The total cell-associated fluorescence was determined as previously described.

Mutagenesis

Cells were grown to a density of 2×10^6 cells/ml in HL5. 2×10^8 cells were resuspended in 10 ml of 20 mM phosphate buffer which contained 1 mg/ml N-methyl-N'-nitro-N-nitrosoguanidine (Sigma Immunochemicals) (Loomis, 1987). The cells were shaken at 150 rpm for 30 min at 21°C, and then washed twice in 40 ml cold HL5 before being resuspended in 50 ml fresh HL5. Cells were allowed to recover for 60 h before mutant enrichment. The survival rate as determined by plating efficiency was ~10%.

Enrichment for Phagocytosis Mutants

Mutagenized cells (1.5×10^6 cells/ml) were resuspended to 4×10^6 cells/ml in 5 ml fresh HL5 with fluoresceinated, heat-killed *K. aerogenes* (5×10^9 bacteria/ml). The suspension was shaken at 150 rpm for 30 min, after which *Dictyostelium* were separated from noninternalized bacteria. Cells were resuspended to 3×10^6 cells/ml in cold HL5 and passed through a fluorescence-activated cell sorter (FACS®, FACS-Star®; Becton-Dickinson Immunocytometry Systems, Mountain View, CA) under sterile conditions a rate of 1,000/s. The cells were excited at 488 nm, and the emission was collected through a 530/30 band pass filter. The least fluorescent 3% of cells were collected. Cells that had been incubated with fluorescent bacteria in the presence of 10 mM NaN₃ were used as a negative control. The collected cells were resuspended in 6 ml HL5 and grown to a density of 4×10^6 cells/ml over 7 d and then subjected to a second round of FACS®.

Visual Screen for Mutants

After the second round of enrichment, cells were clonally diluted into 96-well plates (200 µl HL5/well) and grown for 1 wk. Clones were transferred to 24-well plates and incubated for 30 min with FITC–*S. minnesota Re595* (5×10^9 bacteria/ml) in suspension at 21°C. Noningested bacteria were removed, and the cells observed on a 24×60 mm no. 1 glass coverslip using an Axiovert inverted fluorescence microscope at 630× final magnification. Colonies containing predominantly nonfluorescent cells were recloned before further analysis.

Linkage Analysis

To cross cell lines, the method of Loomis was used with slight modifications (Loomis, 1987). Briefly, cells grown to 1–2 $\times$ 10⁶ cells/ml in HL5 were resuspended at 1 $\times$ 10⁷ cells/ml in phosphate buffer. Equal numbers (5 $\times$ 10⁶) of two mutant cell lines were mixed together and incubated for 15–18 h at 21°C in 24-well plates with constant agitation at 150 rpm. After this overnight incubation, cells were placed under the appropriate selection conditions; crosses between the dysphagia mutants were grown in a suspension of *K. aerogenes* at 21°C, while the crosses between the dysphagia mutant and the temperature-sensitive mutants were grown in suspensions of *K. aerogenes* at 26.5°C. Any cells that survived the selection were cloned on bacteria.

Fluorescence Microscopy

Cells processed using the agar overlay technique (Fukui et al., 1987) were

fixed in 2% paraformaldehyde (PFA)[1] in HL5 for 5 min followed by 5 min at -10°C in 1% PFA in methanol. Alternatively, cells were fixed in suspension with 2% PFA for 5 min and permeabilized with 0.1% Triton X-100 for an additional 5 min. To visualize actin filaments, cells were incubated for 30 min with 0.5 µM rhodamine phalloidin (Sigma Immunochemicals) in PBS. Photographs were taken using a Nikon microscope with a 100× oil immersion objective (final magnification = 1,000) on TMax film (ASA 400; Eastman Kodak Co., Rochester, NY).

Quantitation of Bacterial Binding

Fluoresceinated *S. minnesota Re595* or *K. aerogenes* (5×10^9 bacteria/ml) were incubated for 30 min with *Dictyostelium* (4×10^6 cells/ml) in phosphate buffer at 4°C or in the presence of 10 mM NaN₃. Depending on the experiment, cells were fixed for 10 min with 2% PFA in HL5 either before or after washing away excess bacteria. Cells were resuspended in 100 µl Mowiol, mounted underneath a glass no. 1 coverslip, and observed at a magnification of 1,000 using a Nikon fluorescence microscope. Total number of bacteria/cell was determined by counting at least 100 cells.

Results

Characterization of Phagocytosis by Dictyostelium

To establish optimal conditions for the enrichment of phagocytosis-defective mutants, it was first necessary to characterize bacterial uptake by suspension cultures of *Dictyostelium* cells. Several different strains of bacteria were used. The amebae were incubated with different concentrations of FITC-labeled *S. minnesota Re595* in phosphate buffer at 21°C. At various time points, extracellular bacteria were removed by centrifugation, and the number of cell-associated bacteria were determined. As shown in Fig. 1, phagocytosis was linear at all concentrations of bacteria for the first 30 min
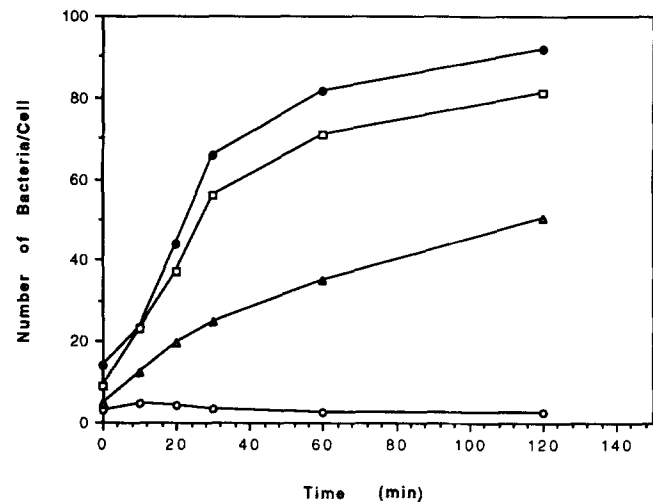

Figure 1. Phagocytosis of *S. minnesota Re595*. Parental *Dictyostelium* strain AX4 (4×10^6 cells/ml) were incubated in 20 mM phosphate buffer pH 6.3 with FITC-labeled *S. minnesota Re595* at the following concentrations: (●) 5.0 × 10⁹ bacteria/ml; (□) 2.5 × 10⁹ bacteria/ml; (△) 1.25 × 10⁹ bacteria/ml; and (○) 2.5 × 10⁹ bacteria/ml + 10 mM NaN₃. At the indicated times, aliquots were removed from suspension, phagocytosis was halted with cold phosphate buffer, and *Dictyostelium* were washed free of noningested bacteria by differential centrifugation. The total number of cell-associated bacteria was determined by fluorimetry.

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1. *Abbreviation used in this paper*: PFA, paraformaldehyde.

and leveled off at longer incubation times. *Salmonella* concentrations of $>2.5 \times 10^9$ bacteria/ml appeared to be saturating for uptake with an initial internalization rate of approximately two bacteria per minute (Fig. 1). Uptake was prevented by incubating cells in 10 mM NaN_3 or at 4°C, indicating that the assay measured phagocytosis rather than the binding of bacteria to cells. Examination by electron microscopy confirmed that virtually all cell-associated bacteria had been ingested (not shown). Comparable results were obtained using FITC-labeled *K. aerogenes* or when the assay was performed in HL5 growth medium.

Isolation of Phagocytosis-defective Mutants

Using these conditions, we next sought to isolate mutants defective in the phagocytosis of FITC-labeled bacteria. *Dictyostelium* cells were mutagenized with nitrosoguanidine to a survival rate of ∼10%. 1.3×10^6 survivors were then incubated with FITC-labeled *K. aerogenes* for 30 min, washed, and then passed through a fluorescence-activated cell sorter (FACS®). The position of nonphagocytic cells was determined on the FACS® profile by sorting parental *Dictyostelium* incubated with labeled bacteria in the presence of NaN_3. The least fluorescent 3% of the population was collected (Fig. 2 *A*), and it was allowed to recover in HL5 for 1 wk. The screen was then repeated, and the 1% least fluorescent cells were collected.

Individual colonies were then visually screened for defects in the uptake of a second strain of bacteria, FITC-labeled *S. minnesota Re595*, which is likely to bind to a receptor distinct from *K. aerogenes*. 7 of the ∼1,500 colonies examined exhibited extreme defects in phagocytosis; we refer to these as "dysphagia" mutants because of their inability to eat bacteria. The phagocytic phenotype of a representative mutant cell line, dysphagia-1, is evident in Fig. 3. After a 30-min incubation in suspension with fluorescent *Salmonella*, dysphagia-1 cells accumulated few if any bacteria; fluorescent bacteria in the field were extracellular. In contrast, parental AX4 cells incubated under identical conditions accumulated multiple FITC-*Salmonella*. This demonstrates that the mutants phagocytose substantially fewer bacteria than parental cells.

Similarly, when bacterial uptake was measured using the quantitative assay, none of the seven mutants were able to ingest significant numbers of bacteria, while the parental AX4 strain internalized the target particles efficiently (Fig. 4). Inhibition of phagocytosis was essentially quantitative because bacterial uptake by the mutant cell lines was not significantly greater than that exhibited by AX4 cells incubated with bacteria on ice. For comparison, we also measured bacterial uptake in a set of mutants previously enriched for phagocytosis and motility defects by resistance to BUdR-labeled *E. coli* at 26.5°C (Clarke, 1978; Kayman et al., 1982). Of the 13 cell lines analyzed, all but one (Fig. 4, *MC6*) were found to exhibit normal levels of phagocytosis at either the permissive or restrictive temperatures (not shown). MC6, however, displayed a maximal defect in phagocytosis (∼70% inhibition) after incubation at the nonpermissive temperature for 20 h (Fig. 4). Shorter shifts of 2–10 h resulted in only moderate internalization defects (not shown).

To ensure that the phagocytosis defects affected all of the cells in the population uniformly, we also analyzed the mutant cells by FACS®. As shown for one cell line, dysphagia-1
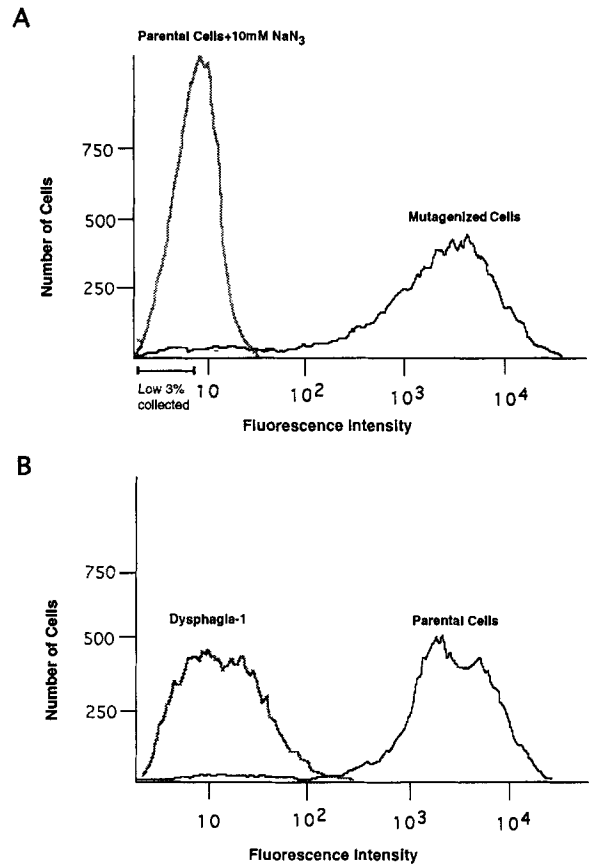
Figure 2. FACS® analysis of mutagenized cells. (*A*) Mutagenized cells (4 × 10⁶ cells/ml) were incubated with fluorescent *Klebsiella aerogenes* (5.0 × 10⁹ bacteria/ml) for 30 min. After the removal of noningested bacteria, cells were passed through a fluorescence-activated cell sorter (FACS®), and the least fluorescent 3% of the cells were collected. Parental cells incubated with bacteria in the presence of 10 mM NaN_3 were used for comparison purposes. (*B*) Dysphagia-1 and parental cells were analyzed in the same way to determine the overall phagocytic capability of the mutant cell population.

(Fig. 2 *B*), all of the mutant cells were recovered in a single peak with mean fluorescence ∼1,000-fold lower than the parental AX4 strain. Interestingly, a small fraction of parental *Dictyostelium* cells exhibited a level of fluorescence comparable to the mutant, suggesting that a subpopulation of cells were unable to phagocytose bacteria at a given point in time. Conceivably, this population may represent cells in mitosis that may be incapable of bacterial uptake.

Phagocytosis-defective Mutants Exhibit Growth Defects on Bacteria but not in Liquid Culture

To determine if the phagocytic defect prevented the mutant cells from surviving under conditions in which growth was entirely dependent on phagocytosis, heat-killed suspensions of *K. aerogenes* were inoculated with either parental or mutant cells. The parental cells (AX4) doubled every 6–7 h and reached densities of ∼2 × 10⁷ cells/ml. The mutant cell lines, however, doubled only once every 24–48 h and attained maximum concentrations of just 2.5–5 × 10⁵ cells/ml after 3 d (Fig. 5 *A*). When incubated for longer
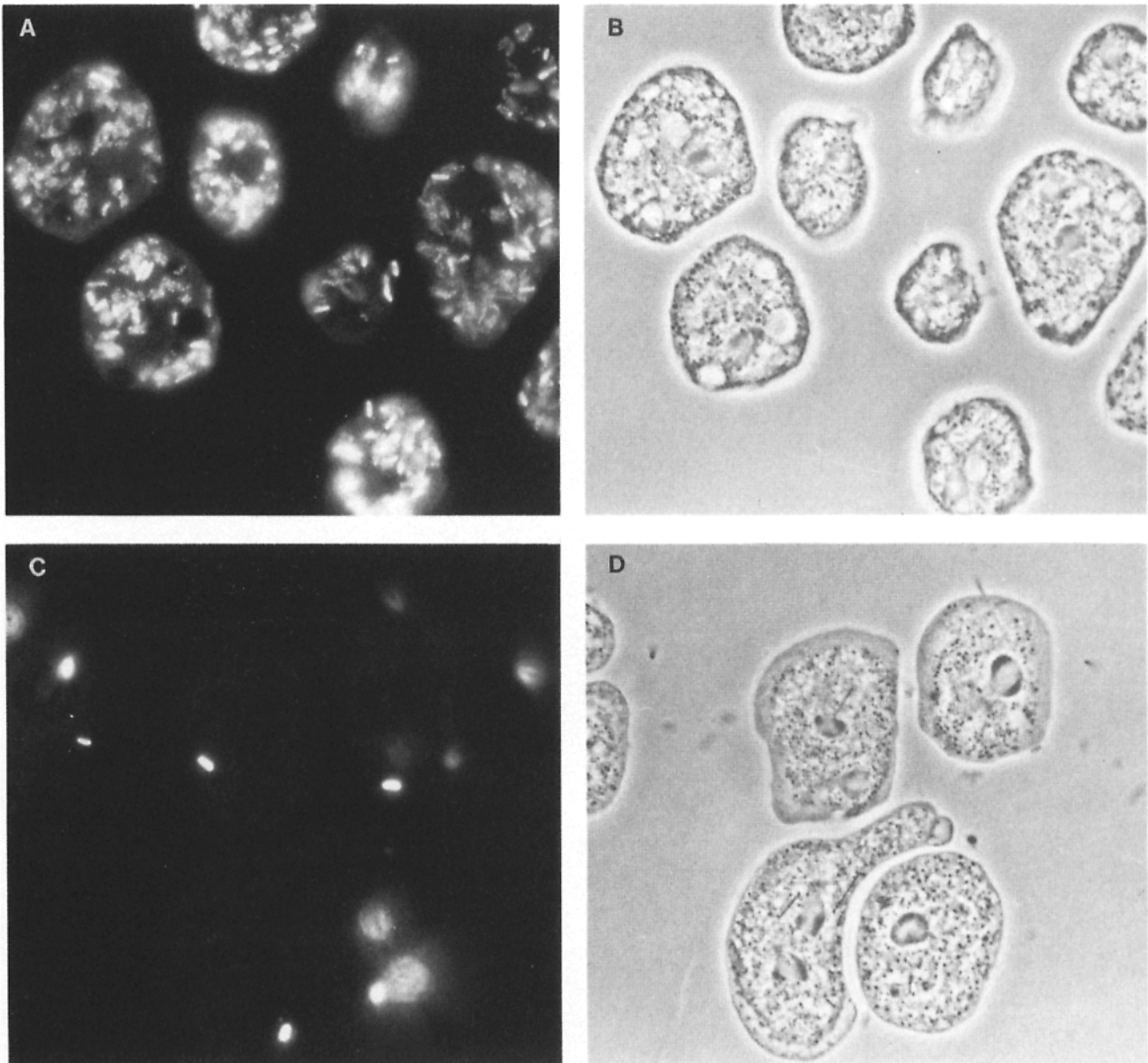
Figure 3. Parental but not mutant cells ingest FITC–*S. minnesota Re595*. Parental (*A* and *B*) and dysphagia-1 mutant cells (*C* and *D*) were incubated with FITC–*S. minnesota Re595* (5.0 × 10⁹ bacteria/ml) for 30 min. Noninternalized bacteria were removed, and the cells were processed by the agar overlay technique. Fluorescent images are shown in *A* and *C*, while phase is displayed in *B* and *D*.

periods of time, the mutant cells died. Similar results were obtained when cells were cultured on heat-killed *S. minnesota Re509* (not shown), indicating that the impairment in growth is not specific to one species of bacteria. These results suggest that defects in phagocytosis prevent the mutants from using a suspension of bacteria as an adequate food source.

The parental AX4 *Dictyostelium* strain used for these experiments is axenic and can grow in liquid culture (HL5), from which it appears to obtain nutrients by fluid-phase endocytosis. Therefore, cells were cultured in HL5 to establish whether the decrease in phagocytosis and growth was caused by a general inhibition of cellular metabolism. As can be seen in Fig. 5 *B*, mutant and parental cells multiplied in HL5 at identical rates, with a doubling time of ~10 h. Therefore, the mutant phenotype cannot be secondary to an overall dim-

inution of cellular function. Because fluid-phase endocytosis of FITC-dextran was also normal in all of the mutants (not shown), the defect in phagocytosis selectively ablated only one limb of the endocytic pathway.

In addition to the dysphagia mutants, we analyzed the growth characteristics of the previously isolated motility mutant, MC6 (Kayman et al., 1982), found to have a temperature-sensitive defect in phagocytosis (Fig. 4). As shown previously, this cell line was unable to grow on bacteria at 26.5°C in either a suspension or on agar plates. However, unlike the mutants isolated here, MC6 was also incapable of axenic growth at the nonpermissive temperature (not shown), although they grew slowly in axenic culture at 21°C. Therefore, unlike the dysphagia mutants, the growth defect in these cells is not specifically caused by an inability to ingest a bacterial food source.
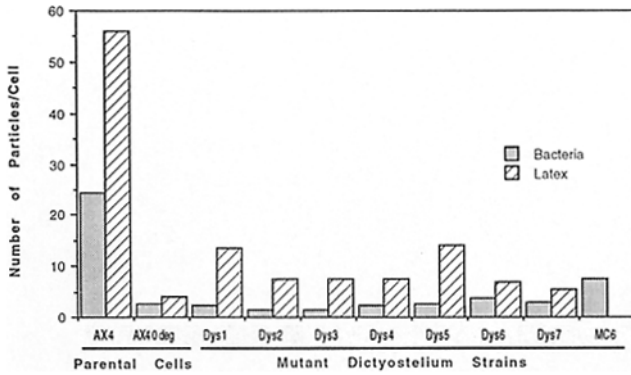
Figure 4. Seven mutant cell lines do not phagocytose bacteria or latex beads. Parental and mutant cell lines were incubated in 20 mM phosphate buffer pH 6.3 with FITC *S. minnesota* Re595 (■) or 1 μm polystyrene latex beads (▨) for 30 min. The concentration of both the bacteria and the latex beads was 5.0×10^9 particles/ml. The total number of internalized particles was determined as outlined in Materials and Methods.
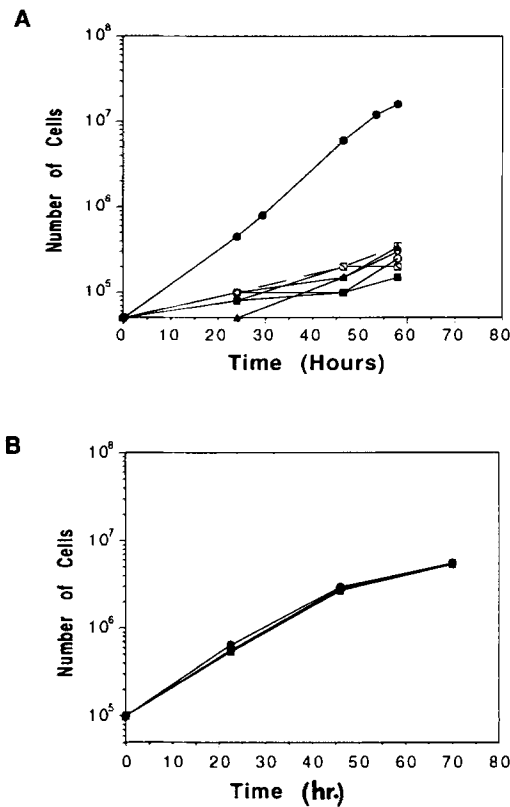

Figure 5. Mutant cells cannot grow in a suspension of bacteria. (*A*) Mutant and parental cells at a starting concentration of 5×10^4 cells/ml were cultured in a suspension of heat-killed *Klebsiella aerogenes.* The cell density was determined at the indicated times. The following cell lines were assayed for growth: (●) AX4 parental, (□) dysphagia-1, (○) dysphagia-2, (◇) dysphagia-3, (▧) dysphagia-4, (△) dysphagia-5, (■) dysphagia-6, and (▲) dysphagia-7. (*B*) Mutant and parental cells were cultured in axenic growth media (HL5) at a starting concentration of 1×10^5 cells/ml.

Phagocytosis Defects Do Not Reflect Altered Binding of Phagocytic Particles

A defect in a phagocytic receptor could cause the decrease in bacterial uptake displayed by the mutant cells. Assuming that there were multiple potential receptors, such a defect might manifest itself as an inability to ingest some, but not all, potential phagocytic particles. Indeed, the phagocytosis mutants described by Vogel et al. (1980) were found only to be defective at internalizing bacteria that lacked glucose moieties on their surfaces. In addition, it has been suggested that receptors recognizing mannose and *N*-acetylglucosamine residues are also present on the plasma membrane of *Dictyostelium* cells (Bozzaro and Roseman, 1983; Cohen, C. J., and I. Mellman, unpublished results). To determine whether our mutants might also be defective at internalizing only certain types of particles, we next assayed phagocytosis using a panel of *E. coli* and *Salmonella* strains with known variations in the lipopolysaccharide structure. Several strains had exposed glucose residues, but none secreted capsular material that would block uptake (see Materials and Methods for a list of the lipopolysaccharide structures).

As shown in Fig. 6, a representative mutant, dysphagia-1, was unable to phagocytose any of the bacterial species that were avidly internalized by the parental AX4 cells. Since the defect in phagocytosis inhibited the uptake of a wide range of bacterial strains, regardless of the accessibility of their surface glucose residues, it was unlikely that the mutations affected bacterial recognition. This also demonstrated that the dysphagia cell lines were distinct from the binding mutants described previously (Vogel et al., 1980).

As an even more stringent test of whether our mutants exhibited a defect in binding, we next determined their ability to ingest polystyrene latex beads. The binding and phagocytosis of latex by both *Dictyostelium* and mammalian cells is thought to reflect nonspecific interactions rather than a specific receptor. When assayed in either phosphate buffer or HL5, parental cells avidly internalized 1-μm polystyrene latex beads, while only a small number of particles were in-
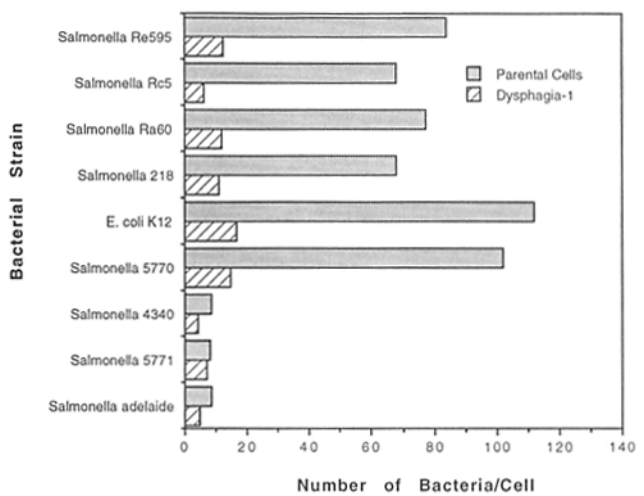

Figure 6. Phagocytosis defect is not dependent on bacterial LPS structure. Dysphagia-1 (▨) and parental cells (■) were incubated with the indicated bacterial strains for 30 min. LPS structures are given in Materials and Methods.

gested by the mutants (Fig. 4). Therefore, the mutation most likely does not affect the binding properties of a specific bacterial receptor.

Finally, to directly determine if the mutants were defective in the binding of bacteria, dysphagia-1 cells were incubated with fluoresceinated *S. minnesota Re595* in the presence of NaN_3 or at 4°C to prevent phagocytosis but not attachment. Cells were fixed with paraformaldehyde, and excess bacteria were removed by centrifugation. Both mutant and parental lines bound approximately two to three *S. minnesota Re595* per cell. Comparable results were obtained when the bacteria were removed before fixation, as well as when the experiment was performed using *K. aerogenes* instead of *Salmonella*. In addition, when cells were fixed and sectioned for EM after a standard 30-min uptake assay, both parental and mutant cells had an equal number of bacteria attached to their surface, although only parental cells had ingested the microorganisms (not shown). Therefore, there does not seem to be a substantial difference in the binding capacity of mutant and parental cells for bacteria.

Phagocytosis-defective Mutants Exhibit Reduced Adherence

Although none of the mutants was found to have a defect in binding phagocytic particles, one striking phenotype was their inability to attach firmly to plastic tissue culture dishes. Homotypic binding between individual amebae was unaffected, however, and in either phosphate buffer or HL5, all of the dysphagia mutants tended to form large aggregates of cells that floated freely in the media. Agglutination was completely inhibited by the addition of 1 mM EDTA, suggesting that it was mediated by contact site B, a *Dictyostelium* plasma membrane protein well known to control Ca^{++}-dependent cell–cell interactions (Beug et al., 1973). In contrast, parental cells adhered firmly to tissue culture plastic. Therefore, it appeared that the mutants have a deficiency in adhesion, although they retain the ability to bind to each other during vegetative growth.

Adherence Reverses the Phagocytosis Defect

Culture conditions have been shown to dramatically influence the growth and functional characteristics of *Dictyostelium* cells. For example, when axenic strains such as AX4 are transferred from a bacterial food source to liquid broth, they increase their capacity for fluid phase endocytosis by >100-fold (Kayman and Clarke, 1983). *Dictyostelium* harboring a deletion of the myosin II heavy chain gene cannot divide in suspension, but can when attached to plastic via "traction-dependent" cytokinesis (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). Although the mutant cells that we have isolated were unable to adhere to tissue culture plates, they were capable of attaching to a solid substrate when cultured on an agar plate or polycarbonate filter. Accordingly, we next determined whether the phagocytosis, growth, and development phenotypes of our mutants might also be controlled by culture conditions.

When cells were cocultured with bacteria on SM-1 agar plates, we found that all the dysphagia mutants cleared the plates of bacteria as rapidly as parental cells. In all cases, the bacteria were ingested within 72 h (not shown). After the clearance of bacteria from the plates, the *Dictyostelium* de-
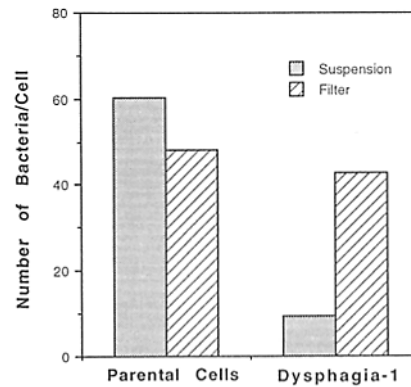

Figure 7. Mutant cells ingest bacteria when incubated on a polycarbonate filter. 1.0×10^7 dysphagia-1 or parental cells were deposited onto a polycarbonate filter in conjunction with 5×10^9 FITC *S. minnesota Re595* in a total volume of 100 μl 20 mM phosphate buffer pH 6.3 (⊠). The mixture was incubated at 21°C for 30 min in a humidified chamber. Cells were resuspended in cold phosphate buffer, and the number of internalized bacteria was determined as previously described. Parental and mutant cells were also incubated in a suspension of bacteria for comparison purposes (■). The other six dysphagia mutants displayed similar uptake characteristics to dysphagia-1 when incubated on polycarbonate filters.

velopmental cycle was initiated because of starvation. The mutant cells developed completely with the formation of fruiting bodies containing viable spores. Cells obtained from these spores were still defective in phagocytosis. Therefore, it is evident that phagocytosis, growth, and development of the dysphagia mutants occur normally under conditions in which the cells are adherent, such as on the surface of an agar plate.

To directly measure phagocytosis by adherent cells, mutants and bacteria were incubated together on polycarbonate filters. As shown in Fig. 7, a representative mutant, dysphagia-1, demonstrated no deficit in the uptake of fluoresceinated *S. minnesota Re595* under these conditions, even though cells assayed simultaneously in a suspension culture were unable to ingest the same target particle. Comparable results were obtained when the other six dysphagia mutants were assayed on filters (not shown). Parental cells, on the other hand, internalized bacteria with equal efficiency either in suspension or on filters. In addition, phagocytosis of the bacteria by the mutants commenced immediately upon contact with the filter, suggesting that the induction of new proteins was not necessary for uptake. Therefore, it appears that this conditional mutation only inhibited phagocytosis by cells in suspension.

Genetic Analysis of Phagocytosis-defective Mutants

One of the advantages of using *Dictyostelium discoideum* for the study of phagocytosis is that they are far more amenable to genetic manipulation than are mammalian phagocytes. These methods allow for the isolation of spontaneous revertants, as well as the placement of different mutants into complementation groups (Loomis, 1987). Therefore, to determine if the seven mutants that we have isolated carry mutations in the same gene, we fused the various mutant cell lines to perform a genetic cross. In addition, we crossed dysphagia-1 with both MC6, the temperature-sensitive phago-

Table I. Genetic Crosses Are Not Defective in Growth, Phagocytosis, or Adherence

	Phagocytic uptake (bacteria/cell)	Doubling time in bacterial suspension (h)	Pinocytosis of FITC-Dextran (percent of parental cells)
Parental Cells (AX4)	43.7 ± 12.9	5.8	100%
Dysphagia-1	3.4 ± 2.5	33.0	118%
MC6 (26.5°C)	6.3 ± 2.5	Nonviable	NA
Indy1 (26.5°C)	33.0 ± 7.4	Nonviable	14%
Dysphagia-1xIndy1 (26.5°C)	43.9 ± 3.7	5.8	89%
Dysphagia-1xMC6 (26.5°C)	24.1 ± 9.7	10.6	NA

Growth in a bacterial suspension, phagocytosis, and adherence were all measured as previously described. NA, not applicable.

cytosis mutant of Clarke (Clarke, 1978; Kayman et al., 1982), as well as indy-1, a mutant with severe temperature-sensitive defects in fluid-phase pinocytosis but normal phagocytosis (Bacon, R. A., C. J. Cohen, D. Lewin, and I. Mellman, manuscript submitted for publication, 1994).

After fusing cells using standard procedures (Loomis, 1987), crosses were selected by incubating the cells in a shaking culture of heat-killed *K. aerogenes* at 26.5°C, conditions under which neither the dysphagia mutants nor the two temperature-sensitive cell lines could survive. After 1 wk, viable cell lines were isolated between dysphagia-1 and indy-1, as well as between dysphagia-1 and MC6, at frequencies of at least 2×10^{-7}. No cells grew from the fusions between the different dysphagia mutants, nor were viable cells recovered when the various mutant cell lines were fused only with themselves. The reversion frequency of these cell lines ($<2 \times 10^{-9}$) was significantly lower than the frequency at which viable complementing "hybrids" were recovered (see the next section). Thus, it appears that the conditional endocytosis defects exhibited by dysphagia-1, indy-1, and MC6 are caused by mutations in separate genes. Since each of these cell lines were found by FACS® to have only haploid genomes, stable diploids were not formed as a consequence of the cell fusion procedures that were used. As a result, it was impossible to determine whether the seven dysphagia mutants tested were contained within single, multiple, or genetically linked complementation groups.

Next, we analyzed the phagocytosis, growth, adherence, and pinocytosis characteristics of the two complementing cell lines that were isolated. As summarized in Table I, all of the defects associated with the mutant cell lines were complemented in the viable crosses. For example, the indy-1 X dysphagia-1 cell line exhibited normal phagocytosis in suspension, as well as normal pinocytosis after 2 h at 26.5°C (conditions that inhibit pinocytosis in indy-1 by >95%) (Bacon, R. A., C. J. Cohen, D. Lewin, and I. Mellman, manuscript submitted for publication). Growth defects and adherence defects were also corrected in both cell lines. Thus, crossing dysphagia-1 with either indy-1 or MC6 coordinately corrected all of the phenotypes associated with the mutations of both parental cells, as would be expected for the genetic complementation of single gene defects.

The theory that the dysphagia mutants resulted from single genetic events was further supported by the fact that it was possible to isolate a revertant of one cell line, dysphagia-6, based on its ability to grow in a suspension of heat-killed *K. aerogenes*. The frequency of reversion was ~5.0 × 10^{-8}. The revertant cells displayed parental levels of phago-

cytosis, growth in suspension, and adherence (Table II). No revertants were recovered from any of the other cell lines even after culturing >5 × 10^8 cells, indicating that reversion frequencies for these dysphagia mutants were <2 × 10^{-9}. The fact that the revertant regains the capacity for both phagocytosis and adherence implies that the defects exhibited by the mutant are caused by a single genetic event.

Dysphagia Cells Fail to Trigger the Actin Polymerization Necessary for Phagocytosis

Dictyostelium have an actin-rich cytoskeleton that is intimately involved in the phagocytic ingestion of microorganisms and other particles. Therefore, we probed both mutant and parental cells with rhodamine phalloidin to determine if actin polymerization occurred normally in the dysphagia mutants. All cell lines were fixed and stained in suspension after incubation with FITC–*S. minnesota* Re595. In the parental cells, actin concentrated within the phagocytic cup surrounding bacteria that were being actively ingested (Fig. 8 *B, open arrows*), which is comparable to what has been seen in past studies of actin dynamics during phagocytosis in both mammalian cells and *Dictyostelium* (Fukui et al., 1989; Greenberg et al., 1990). In contrast, no actin staining was visible around any of the bacteria associated with dysphagia-1 (Fig. 8 *D*) or any of the other dysphagia mutants (not shown). Nevertheless, distinct cortical actin fluorescence was evident in both parental and mutant cells (Fig. 8, *B* and *D*), which implies that actin assembly and stability are unaffected in the dysphagia mutants. Because there was no significant difference in the overall morphology of the actin based cytoskeleton when compared with parental cells, the dysphagia mutants appear to lack the ability to trigger the localized actin polymerization necessary for phagocytosis to occur when cultured in suspension. This implies that the mutation may lie somewhere along the signal transduction path-

Table II. Revertant Is Not Defective in Growth, Phagocytosis, or Adherence

	Phagocytic uptake (bacterial/cell)	Doubling time in bacterial suspension (h)
Parental Cells (AX4)	65.3 ± 14.0	6.7
Dysphagia-6	8.1 ± 0.1	40.0
Revertant	42.9 ± 14.2	6.7

Growth in a bacterial suspension, phagocytosis, and adherence were all measured as previously described.
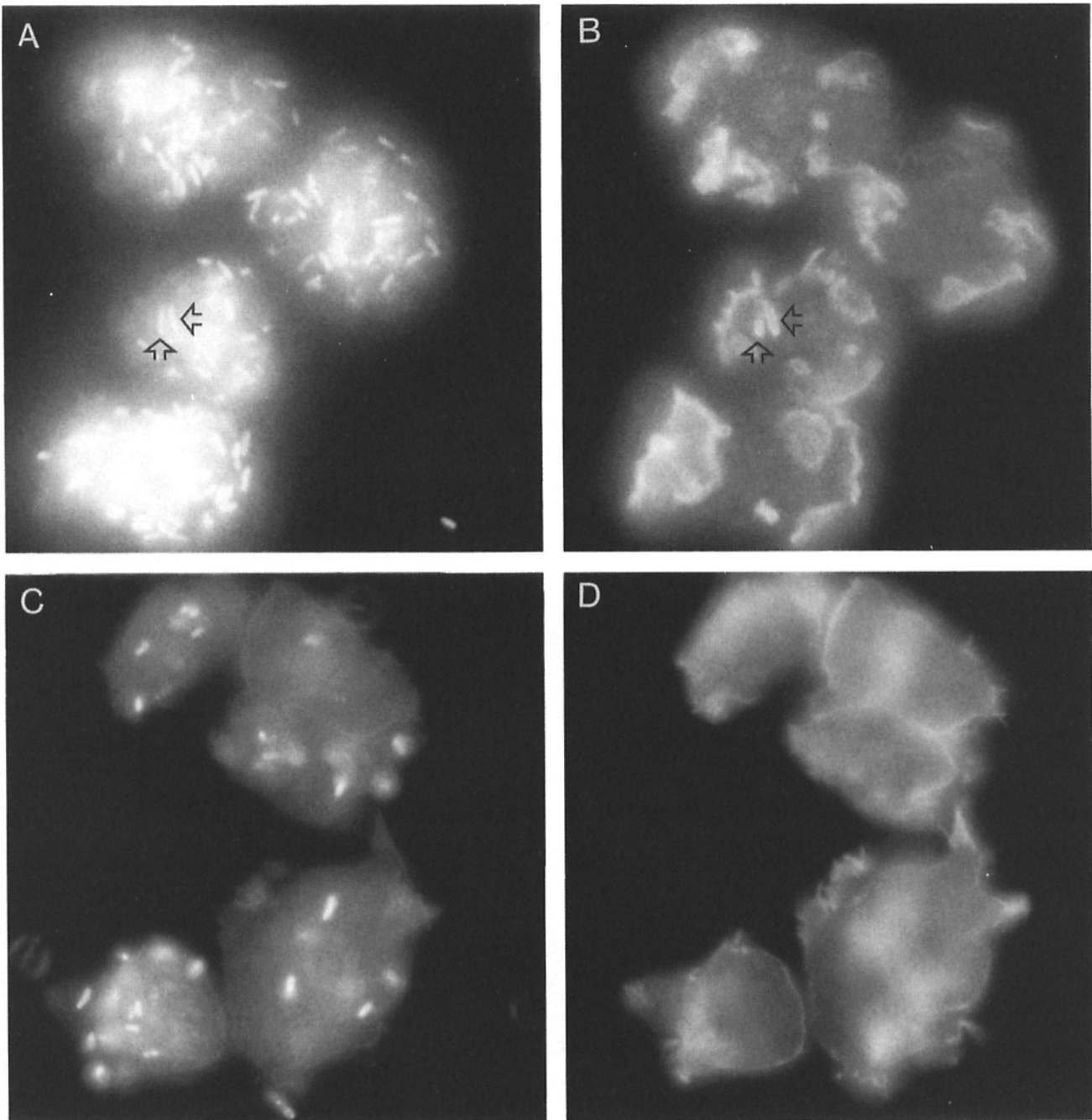
Figure 8. The morphology of the actin-based cytoskeleton is similar in mutant and parental cells. Parental (*A* and *B*) and dysphagia-1 cells (*C* and *D*) were incubated with FITC–*S. minnesota Re595* (5.0 × 10⁹ bacteria/ml) for 10 min. Cells were subsequently stained with rhodamine phalloidin to visualize actin filaments. *A* (parental) and *C* (dysphagia-1) show FITC-labeled bacteria while *B* (parental) and *D* (dysphagia-1) display rhodamine phalloidin-labeled actin filaments. Bacteria that are being actively ingested are indicated by open arrows in *A* and *B*. The other six dysphagia mutants behaved in a fashion similar to dysphagia-1.

way responsible for initiating the ingestion process after particle binding.

Discussion

We have initiated a genetic approach to the study of phagocytosis by screening for and analyzing phagocytosis-defective mutants of *D. discoideum. Dictyostelium* is a well-characterized, protozoan ameba that normally relies on the phagocytic uptake of bacteria as its sole food source. Importantly, phagocytosis in *Dictyostelium* cells bears many critical features in common with phagocytosis in mammalian leukocytes. While phagocytosis in the two cell types must rely on different arrays of cell surface receptors, these receptors must be related at least in their ability to transduce signals that initiate a common phagocytic response. It is possible that at least some phagocytic receptors in mammalian cells are even structurally related to phagocytic receptors in *Dic-*

tyostelium, since both cell types are known to possess receptors with mannose- or glucose-specific lectin activities (Bozzaro and Roseman, 1983; Lennartz et al., 1987a, 1987b; Vogel et al., 1980; Cohen, C. J., and I. Mellman, unpublished results). Taken together, these considerations suggest that phagocytosis is an evolutionarily conserved function that should prove amenable to genetic analysis in simple organisms such as *Dictyostelium*.

Previous attempts to isolate phagocytosis-defective *Dictyostelium* mutants have met with limited success, yielding cells with partially defective phenotypes or with defects in bacterial binding as opposed to internalization (Kayman et al., 1982; Vogel et al., 1980). Since earlier attempts relied on the use of indirect screens, we developed a simple and direct strategy that permitted the isolation of mutants with severe defects that would prove lethal for cells propagated on bacteria alone. We made use of the fact that axenic strains of *Dictyostelium* grow equally well when cultured on a bacterial food source or in liquid broth. Since the *Dictyostelium* plasma membrane has limited capacity for nutrient transport (North, 1983; Turner et al., 1979), growth on bacteria is completely dependent on phagocytosis, while growth in liquid media is dependent on pinocytosis of extracellular fluid. Thus, liquid medium should represent a permissive condition for the growth of cells with severe defects in phagocytosis that would be lethal for growth on bacteria.

Using this approach, seven dysphagia mutants that exhibited virtually complete conditional defects in bacterial phagocytosis were isolated. Since each cell line was unable to internalize a wide variety of bacteria with different surface coats, as well as nonspecifically-bound latex beads, it was apparent that the mutations affected some intracellular event required for phagocytosis rather than simply particle attachment. In addition, when measured directly, the dysphagia mutants exhibited no deficit in the binding of bacteria. Suspension cultures of the defective cells were unable to grow on bacteria, but they exhibited normal growth in liquid medium. Pinocytosis was normal in all of the mutants. An additional defect was that the mutant cells could not firmly adhere to tissue culture dishes. Nevertheless, when cultured on agar plates or polycarbonate filters, the dysphagia mutants were able to phagocytose, grow, and develop normally. Revertants that corrected the deficits in phagocytosis, adherence, and bacterial growth were also isolated, suggesting that they all reflected a mutation affecting a single gene. Finally, no alterations in the cytoskeleton of the mutant cells that could account for the dramatic conditional defect in phagocytosis exhibited by these cells were identified. Thus, it appears that the mutant phenotype may reflect an inability of the dysphagia cells to couple bacterial binding with the generation of a signal that triggers the internalization of the bound particle.

Unfortunately, it proved impossible to determine whether the seven dysphagia mutants represented unique or overlapping genetic complementation groups because we were unable to generate stable diploid cell lines using classical techniques for fusion of *Dictyostelium* cells. Thus, the lack of complementation might simply reflect a low fusion efficiency or the aberrant segregation of chromosomes containing the parental copies of each gene rather than all seven mutants belonging to a single complementation group. Since somatic recombination is a rare event in *Dictyostelium*, complemen-

tation also would not have been easily observed for genes in the same linkage group. On the other hand, we cannot rule out that the seven dysphagia mutants represent a single lineage that preferentially grew during the recovery period. It is clear, however, that a phagocytosis-defective phenotype can result from mutations of at least two distinct genes. Parental phenotypes were obtained in haploid segregants recovered from crossing one of the mutants (dysphagia-1) with a previously isolated mutant (MC6) that has a partial temperature-sensitive defect in phagocytosis. MC6 was also phenotypically distinct from dysphagia-1 in that MC6 cells did not exhibit altered adherence properties, and that they were unable to grow in a suspension of bacteria, on a lawn of bacteria, or in liquid medium at the restrictive temperature.

The fact that none of the dysphagia mutants affected fluid phase pinocytosis indicates that these two processes of endocytosis can be distinguished genetically. This is further supported by the fact that we have also been able to isolate mutants with temperature-sensitive defects in pinocytosis which exhibit normal phagocytosis (Bacon, R. A., C. J. Cohen, D. Lewin, and I. Mellman, manuscript submitted for publication). Indeed, fusion of one such pinocytosis mutant (indy-1) with dysphagia-1 was found to yield a stable haploid segregant in which the phenotypic defects of both mutants were completely corrected. Moreover, all aspects of the mutant phenotype in dysphagia-6 were similarly "corrected" in a revertant, further indicating that this mutation resulted from a defect affecting a single gene.

Final characterization of the defects must await the identification of the defective gene(s). However, it is possible to draw some conclusions on the likely nature of the defects. As mentioned previously, the mutation does not appear to have adversely affected the binding of bacteria to the dysphagia cell lines. In addition, the inability of the dysphagia mutants to mediate phagocytosis did not reflect an overall decrease of cell viability. Not only were the cells capable of normal growth in liquid media, but also they were able to "correct" their phagocytosis defect immediately upon attaching to a substrate. This finding is reminiscent of previous results in which *Dictyostelium* cells bearing a deletion of the myosin II gene exhibited a profound defect in cytokinesis in suspension but were able to divide by a modified cell fission event when attached to substrates (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987). Differences in the organization and/or function of the cytoskeleton in suspension vs adherent cells must thus permit the modulation of complex functions such as cytokinesis or phagocytosis. Accordingly, it is possible that one or more dysphagia mutants possess an alteration in a cytoskeletal protein needed for phagocytosis in suspension but not when adherent. However, this seems unlikely for several reasons. First, none of the numerous *Dictyostelium* cytoskeletal gene knockout mutants have exhibited significant defects in either phagocytosis or adhesion, although the myosin I knockout cells of Jung and Hammer did show a slight diminution in phagocytic ability (Andre et al., 1989; Brink et al., 1990; Cox et al., 1992; Gerisch et al., 1991; Jung and Hammer, 1990; Walraff et al., 1986; Witke et al., 1992). In addition, while actin is clearly involved in phagocytosis, this gene is present in multiple copies in *Dictyostelium*, making it difficult to analyze the function of any one isoform in isolation. Finally, examination of

the cytoskeleton of both mutant and parental cells revealed no obvious structural alterations between the cell lines. In fact, the overall cortical actin staining pattern of dysphagia-1 was indistinguishable from that of parental cells when cultured in suspension, implying that actin assembly and stability are not altered in the mutant cell lines. Although we cannot eliminate a lesion in the cytoskeleton as the cause of the mutants' altered phagocytic phenotype, the defect presumably lies elsewhere.

A more likely possibility is that the phagocytosis-defective phenotype reflects a failure in some aspect of signal transduction. Conceivably, bacteria could bind to the mutants in suspension, but ingestion might not be triggered because of an uncoupling of the attachment and internalization steps. In fact, one difference noted between the cell types was that in suspension, the dysphagia mutants were unable to initiate the localized polymerization of actin beneath an attached particle that is necessary for phagocytosis, even though there were no defects detected in the actin-based cytoskeleton as mentioned previously. In contrast, when cultured on filters or agar plates, the mutants may be constitutively activated to phagocytose any particle with which they come into contact. This would be consistent with observations in mammalian cells, suggesting that adherence via integrins may potentiate the activity of distinct phagocytic receptors (Gresham et al., 1991).

In addition, recent work in Dictyostelium cells demonstrating that tyrosine phosphorylation plays an important role in modulating the dynamics of the actin-based cytoskeleton supports the notion that a defect along a signal transduction pathway may lead to the conditional phagocytic capability of the dysphagia mutants. When vegetative parental Dictyostelium were starved by incubating in phosphate buffer for 4 h and then shifted back into a nutrient medium, a number of proteins, including actin, became phosphorylated on tyrosine residues (Howard et al., 1993; Schweiger et al., 1992). Coincident with these phosphorylation events, the cells rounded up and detached from plastic or glass surfaces, thus exhibiting a defective adherence phenotype similar to that characteristic of the dysphagia mutants. Moreover, parental Dictyostelium cells treated in this fashion also lost the ability to mediate phagocytosis in suspension, although they still ingested bacteria when incubated on a polycarbonate filter (Cohen, C. J., and I. Mellman, unpublished results). Again, the phagocytic properties displayed by parental cells treated in this manner closely resemble those of the mutant cell lines. Therefore, the ability to generate a mutant phenocopy from parental cells suggests that some event along the signal transduction pathway linking tyrosine phosphorylation with cytoskeletal dynamics and phagocytosis may be defective in the dysphagia mutants.

To examine this possibility, we performed Western blots of lysates of both mutant and parental cells using an affinity purified rabbit polyclonal antiserum directed against phosphotyrosine (generously provided by Bart Sefton, The Salk Institute for Biological Sciences, San Diego, CA). In our hands, a slight increase in phosphorylation of actin and a protein of ~110 kD was noted in both mutant and parental cells that were transferred from starvation conditions to nutrient media as previously reported (not shown) (Howard et al., 1993; Schweiger et al., 1992). In addition, as originally demonstrated by Schweiger and colleagues, a significant enhancement in phosphorylation of numerous proteins, including actin, occurred among all cell lines after treatment with phenyl arsine oxide, an inhibitor of cellular phosphatases (not shown) (Schweiger et al., 1992). Nevertheless, no difference was consistently detected in the pattern of tyrosine phosphorylation when comparing mutant and parental cell lines. Therefore, the phagocytosis defect exhibited by the dysphagia mutants is not directly caused by an alteration in tyrosine phosphorylation. Nevertheless, a step either downstream of this event or on an intersecting pathway may be altered in a fashion that prevents the proper triggering of phagocytosis after particle binding.

We next intend to use complementation cloning to determine the precise defect(s) responsible for the mutant phenotype. This technique, which has been used extensively in Saccharomyces cerevisiae, has yet to be routinely applied in Dictyostelium although one successful example has thus far been reported (Dynes and Firtel, 1989). Given that the efficiency of transformation of Dictyostelium cells is at least as high as for yeast ($>3 \times 10^{-3}$), it seems very likely that this approach will prove successful. Therefore, it should be possible to select for genetically rescued mutants based upon their ability to survive in a suspension of bacteria after transformation of the mutant cells with a parental genomic (or cDNA) library. Using these genetic methods, we ultimately hope to identify novel elements involved in phagocytosis.

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