

Mechanism of Phagocytosis in *Dictyostelium discoideum*: Phagocytosis is Mediated by Different Recognition Sites as Disclosed by Mutants with Altered Phagocytotic Properties

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ABSTRACT The recognition step in the phagocytotic process of the unicellular amoeba *Dictyostelium discoideum* was examined by analysis of mutants defective in phagocytosis. Reliable and simple assays were developed to measure endocytotic uptake. For pinocytosis, FITC-dextran was found to be a suitable fluid-phase marker; FITC-bacteria, latex beads, and erythrocytes were used as phagocytotic substrates. Ingested material was isolated in one step by centrifuging through highly viscous poly(ethyleneglycol) solutions and was analyzed optically.

A selection procedure for isolating mutants defective in phagocytosis was devised using tungsten beads as particulate prey. Nonphagocytosing cells were isolated on the basis of their lower density. Three mutant strains were found exhibiting a clear-cut phenotype directly related to the phagocytotic event.

In contrast to the situation in wild-type cells, uptake of *E. coli* B/r by mutant cells is specifically and competitively inhibited by glucose. Mutant amoeba phagocytose latex beads normally but not protein-coated latex, nonglycosylated bacteria, or erythrocytes. Cohesive properties of mutant cells are altered: they do not form EDTA-sensitive aggregates, and adhesiveness to glass or plastic surfaces is greatly reduced.

Based upon these findings, a model for recognition in phagocytosis is proposed: (a) A lectin-type receptor specifically mediates binding of particles containing terminal glucose (*E. coli* B/r). (b) A second class of "nonspecific" receptors mediate binding of a variety of particles by hydrophobic interaction. Nonspecific binding is affected by mutation in such a way that only strongly hydrophobic (latex) but not more hydrophilic particles (e.g., protein-coated latex, bacteria, erythrocytes) can be phagocytosed by mutant amoebae.

Endocytosis is the uptake of fluid (pinocytosis) or particles (phagocytosis) by a eucaryotic cell from the extracellular environment into the cytoplasm via plasmamembrane-derived vesicles. Pinocytosis appears to be a constitutive property of many cells and seems to proceed with a basal rate characteristic for each cell type. The factors controlling this basal rate have not been identified. Phagocytosis involves recognition and binding of a particle by the phagocyte. Binding seems to create a transmembrane signal which leads to circumferential attach-

ment of the particle by pseudopodial movement and subsequent internalization by membrane fusion. (For review, see references 1-5). However, the underlying biochemical mechanism by which a particle is attached to the plasmamembrane of a phagocytotic cell and how this subsequently directs the contractile system to engulf this particle remains unclear.

As an experimental system for studying phagocytosis, we have chosen the unicellular slime mold *Dictyostelium discoideum* (6). In nature, this amoeba grows by ingestion of soil

microorganisms. For laboratory use, axenically growing strains are available which can grow by high, continuous rates of pinocytosis but retain the capacity to phagocytose microorganisms (7). Homogeneous populations of amoebae can be grown in large quantities. Moreover, the potential for genetic studies makes this organism particularly attractive (8).

By isolating mutants with altered phagocytotic properties, we have started to dissect the complex process of phagocytosis into individual steps. Analysis of the mutant phenotype reveals the presence of at least two independent receptors on the surface of *D. discoideum* which recognize different surface features on various substrate particles.

MATERIALS AND METHODS

Strains and Growth Conditions

For axenic cultures, strain AX2 (ATCC24397) and derived mutants were grown in peptone-yeast extract medium supplemented with 18 g glucose or maltose/liter (7). Cell number was monitored with a particle counter (model DN, Coulter Electronics Ltd., Harpenden, England). Cells were harvested by centrifuging at 200 g for 4 min. Amoebae were also grown in submerged cultures on a gyratory shaker (120 rpm) in suspensions of *E. coli* B/r (10^{10} cells/ml) or in association with bacteria on nutrient agar plates (1 g glucose, 1 g bacto-peptone, 1 g yeast extract, 10 g agar in 1 liter of 17 mM sodium phosphate, pH 6.2). For preservation of strains, clonally derived spores were suspended in axenic medium and stored in liquid nitrogen. Spores were found to remain viable for several years under these conditions.

Genetic nomenclature is based upon a system proposed for bacterial genetics (9) which was adapted to *D. discoideum* genetics (10). Haploid strains isolated in this laboratory are designated HV and named according to their isolation number. The locus code *tsg* has been used for genes determining temperature sensitivity for growth, and we propose the locus code *phg* for genes determining the phagocytosis phenotype. Each independently isolated mutation is given an isolation number. A block of isolation numbers from 1350 to 1399 has been allocated to this laboratory.¹

Quantitation of Endocytotic Uptake

The major experimental difficulty in measuring initial rates of phagocytosis is to find a rapid procedure to separate quantitatively cells containing ingested material from the bulk of uningested material. Separation by differential centrifugation, the procedure usually applied, is tedious and incomplete. We have overcome this problem by centrifuging the cell suspension through a column of highly viscous solution of poly(ethylene glycol) 6000. Extracellular fluid and small particles like bacteria or latex beads (diameter below 2 μ m), with a high surface to volume ratio, remain on top of the column, whereas the large amoebae are found on the bottom. Cells remain fully viable during this procedure, and recovery is almost 100%.

BACTERIA PHAGOCYTOSIS: Fluorescein-labeled bacteria (FITC-bacteria) were prepared by incubating bacteria ($OD_{420} = 20$) in 50 mM Na_2HPO_4 , pH 9.2, in the presence of 0.1 mg/ml fluorescein isothiocyanate at 37°C for 3 h. To remove surplus reagent, cells were washed by centrifugation until no fluorescence was detectable in the supernate. For the phagocytosis assay, amoebae were harvested and resuspended in the medium specified at a concentration of $2-4 \times 10^6$ cells/ml. Cells were incubated on a rotary shaker (100 rpm) for 15 min to recover and FITC-bacteria (8×10^8 bacteria/ml) were added. Phagocytosis was stopped by diluting 1-ml aliquots at various times into 2 ml of ice-cold 20 mM phosphate, pH 6.2. To separate amoebae from noningested bacteria, the cell suspension was layered over and centrifuged through (200 g, 10 min) an aqueous solution (10 ml, 7 cm height) of 20% (wt/wt) poly(ethylene glycol) 6000. Noningested bacteria remaining in the top fluid layer were removed, and pelleted amoebae were washed once by centrifugation in 3 ml of 50 mM Na_2HPO_4 , pH 9.2, and resuspended in 3 ml of the same buffer. After counting, cells were lysed by addition of Triton X-100 (0.2% final concentration), and fluorescence intensity of the solution was determined (excitation wavelength: 470 nm, emission wavelength: 520 nm). The number of bacteria ingested was determined by comparison with a standard curve obtained by lysing a defined number of bacteria in an SDS solution (1%, 2 min heating at 90°C), and determining the fluorescence intensity in aliquots of this solution diluted in the above buffer. The additional treatment with SDS was necessary because noningested bacteria, in contrast to ingested

ones, are not lysed by Triton X-100. The treatment does not cause a change in quantum yield. Under the experimental conditions described, no quenching by cytoplasmic components has been observed, as the calibration factor was found to be the same in lysed cell medium (pH 9.2) and in Na_2HPO_4 solutions (pH 9.2). The fluorescein fluorescence is very pH sensitive, but all the experiments were performed over a pH range of pH 9-9.2, within which the dye fluorescence is constant and maximal. The dye to bacteria ratio was approximately the same in all FITC batches as indicated by the calibration factor. Furthermore, as a control, ^{35}S -labeled *E. coli* B/r were used as substrate particles. Ingestion rates observed with radioactively labeled bacteria were quantitatively the same as those measured with the use of FITC-labeled bacteria.

LATEX PHAGOCYTOSIS: The phagocytosis assay with monodisperse preparations of polystyrene latex beads (diameter 1.08 μ m, Dow-Latex; Serva, Heidelberg, W. Germany) was performed in exactly the same way as described for bacteria. The number of ingested beads was determined by measuring optical density at 560 nm after lysis of amoebae as described above and comparison with a standard curve. Alternatively, FITC-labeled latex beads (diameter 0.883 μ m; Polysciences, Inc., Warrington, Pa.) can be used and determined fluorimetrically as described above.

Uptake of bacteria and latex beads can be determined simultaneously in the same batch of cells. After incubation as described above and lysis of amoebae with Triton X-100, the number of ingested latex beads can be determined by measuring the optical density. Subsequently, the beads are removed by centrifuging for 10 min at 500 g. Ingested FITC-bacteria are lysed by Triton X-100, and the fluorescence of the supernate is determined.

ERYTHROCYTE PHAGOCYTOSIS: Uptake of sheep erythrocytes was determined as described previously (11). In brief, erythrocytes and amoebae were incubated in axenic medium. Aliquots of 2 ml were taken at various times and diluted fivefold in ice-cold water to lyse noningested erythrocytes. The amoebae were pelleted by centrifugation and dissolved in 2 ml of formic acid. Hemoglobin of ingested erythrocytes was determined by measuring optical density at 420 nm, and their number, was estimated by comparison with a standard curve.

PHAGOCYTOSIS ON FILTERS: Amoebae (5×10^6) and substrate particles (1.5×10^6) were rapidly mixed in 2 ml of the medium specified. The suspension was uniformly deposited on filters (AABP04700, 0.8 μ m, pore size 47 mm diameter; Millipore Corp., Bedford, Mass.) resting on presoaked absorbent support pads. The samples were then incubated in 60-mm plastic petri dishes at the desired temperature in a moist atmosphere. After various times, cells were harvested by placing the filter in a centrifuge tube containing 4 ml of ice-cold medium and resuspending the cells by vigorous shaking. Subsequently, the procedures described above were followed.

Phagocytotic uptake of the various particles is saturable with respect to particle concentration (data not shown). A maximum rate of initial uptake was obtained at a ratio of particles to amoebae of about 200:1. In shaken cultures, a wild-type amoeba ingests about four to eight *E. coli* B/r (cf. Fig. 4), about 8-14 latex beads (cf. Fig. 5), and about 0.2 erythrocytes (cf. Fig. 7) per min. Uptake rates are linear with incubation time for ~8 min with bacteria, ~4 min with latex beads, and ~40 min with erythrocytes. Control incubation at ice-bath temperature or in the presence of an uncoupler of oxidative phosphorylation (cyanide-*m*-chlorophenylhydrazine, 1 μ M) yielded negligible background levels in the case of bacteria and erythrocytes. In contrast, some batches of latex beads yielded relatively high background values. This indicates that mere adsorption of latex beads sometimes interferes with the phagocytosis assay and makes the estimation of truly ingested particles inaccurate.

ASSAY FOR PINOCYTOSIS: FITC-dextran (FITC-dextran 60, Pharmacia, Uppsala, Sweden) was used as a fluid-phase marker. Amoebae were suspended at a density of $2-4 \times 10^6$ cells/ml in axenic medium, and FITC-dextran was added to a final concentration of 2 mg/ml. Pinocytosis was stopped by diluting 1-ml aliquots at various times into 4 ml of ice-cold 20 mM phosphate, pH 6.2. Cells were collected by centrifuging for 5 min at 100 g, resuspended in phosphate buffer, and centrifuged through a poly(ethylene glycol) 6000 solution as described above. After washing once, cells were resuspended in 2 ml of a 50 mM Na_2HPO_4 solution and the cell number was counted. Subsequently, cells were lysed by addition of Triton X-100 (0.2% final concentration), the fluorescence intensity of the solution was measured, and the pinocytosed volume was determined by comparison with a standard curve.

According to the following criteria, FITC-dextran qualifies as a suitable fluid-phase marker: FITC-dextran is nontoxic for the cells and can be analyzed fluorimetrically in small amounts. Uptake of FITC-dextran is directly proportional to its concentration in the medium from 0.5-10 mg/ml (Fig. 1A). This is consistent with a bulk transport of this molecule, because receptor-mediated uptake would be expected to exhibit saturation characteristics. Uptake rate is proportional to cell concentration (Fig. 1B) and proceeds linearly with time for at least 1 h (cf. Fig. 2). Furthermore, no uptake is observed at 0°C, or at 20°C in the presence of an uncoupler of oxidative phosphorylation (carbonyl cyanide-*m*-chlorophenylhydrazine, 1 μ M). Uptake rates obtained with FITC-dextran were identical to those measured with the use of horseradish peroxidase, a well-established fluid phase marker (12).

¹ K. L. Williams, Australian National University, Canberra. Personal communication.

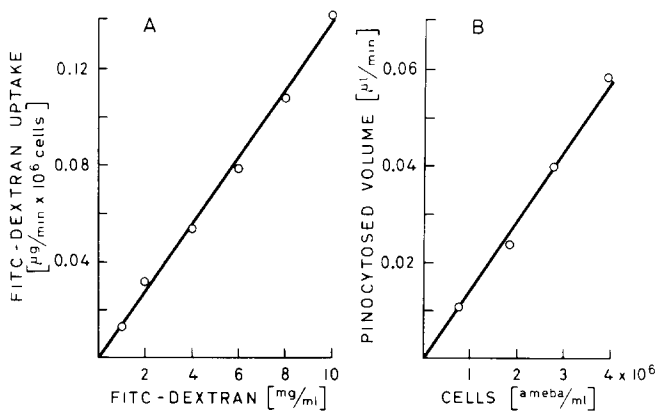


FIGURE 1 (A) Uptake of FITC-dextran by wild-type amoebae at 20°C as a function of its concentration in axenic medium. (B) Fluid uptake by wild-type amoebae in axenic medium at 20°C as a function of cell density.

Mutagenesis

Amoebae grown axenically to a density of $2-4 \times 10^6$ cells/ml were harvested and washed twice with 20 mM potassium phosphate, pH 6.2. They were suspended at a concentration of about 3×10^7 cells/ml in the above buffer. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from a freshly prepared stock solution (50 mg/ml) in dimethyl sulfoxide was added to a final concentration of 1 mg/ml. Cells were incubated on a rotary shaker at 120 rpm at 20°C. After 30 min, the suspension was chilled and cells were washed twice in phosphate buffer. Viability tests revealed a survival rate between 1 and 5%. Immediately after mutagenesis, cells were resuspended in axenic medium at a concentration of about 1×10^6 cells/ml and separated into different batches. Amoebae required 4 d to recover and were subsequently grown at 20°C to a density of $3-4 \times 10^6$ cells/ml, about five to seven doublings. The selection procedure was applied separately to each batch of cells.

Selection Procedure

Mutagenized cells were shifted to 27°C and incubated on a reciprocal shaker at 150 rpm. After 2 h at 27°C, ~200 mg tungsten beads (1 µm diameter; Planseewerke, Plansee, Austria) was added/10 ml of the cell suspension, and the incubation was continued for another 2 h. To remove the bulk of noningested tungsten beads, the incubation mixture was allowed to stand for 5 min without shaking, and the supernatant fluid was carefully decanted into centrifuge tubes. The mixture was diluted 1:3 by addition of axenic medium and centrifuged for 2 min at 70 g in a swing-out rotor to precipitate mainly cells containing tungsten beads. The centrifugation was repeated until no cells containing tungsten beads were detectable microscopically in the supernate. The tungsten treatment was repeated twice with intermittent growth of the amoebae at 20°C. Attempts to facilitate the separation procedure by use of iron or nickel beads and subsequent removal of phagocytosing cells magnetically were not successful. The magnetic particles tended to form clumps during the incubation period and were scarcely phagocytosed. Cells remaining after the tungsten treatment were plated clonally at 20°C on agar plates in association with *E. coli* B/r. Clones were examined for temperature-sensitive growth by transferring cells with toothpicks in duplicate to agar plates previously spread with bacteria and by replica plating at 20° and 27°C. Temperature-sensitive clones were purified twice over single colonies. Only one clone was collected from each batch of cells to ensure that all mutants obtained are of independent origin.

RESULTS

Enrichment of Mutants Defective in Phagocytosis

A strategy for the isolation of mutants in the endocytotic pathway must recognize that pinocytosis and phagocytosis, the two modes of endocytosis, may share common steps (13). Because endocytosis is the sole mechanism of nutrient uptake in *D. discoideum*, mutants defective in steps common to both processes are expected to be lethal. Consequently, a selection scheme was devised to isolate conditional-defective mutants.

Cells of strain AX2 growing exponentially in axenic medium were mutagenized and subsequently incubated in axenic medium in the presence of tungsten beads at 27°C, the nonpermissive temperature. Cells that did not phagocytose tungsten beads could be isolated on the basis of their lower density. The selection is quite effective since only 1-5% of cells, virtually free of tungsten beads, remained after this procedure.

Growth of amoebae on bacteria is dependent upon phagocytosis. Therefore, cells remaining after the tungsten treatment were screened for temperature-sensitive growth at 27°C on nutrient agar plates in association with *E. coli* B/r. About 5-10% of the clones were found to be temperature sensitive for growth. This frequency is about 50 to 100 times that obtained with mutagenized cells when the tungsten treatment is omitted. About 100 independently selected mutants have been isolated.

Phenotypic Classification of Mutants

All mutants are temperature sensitive for growth on bacteria plated on nutrient agar. Although growth on bacteria is dependent upon phagocytosis, defects in a variety of essential cellular functions only indirectly connected to the process of phagocytosis are expected to show this phenotype. To detect mutants directly affected in the phagocytotic process, mutant strains were initially tested for their ability to grow by pinocytosis in axenic medium at the nonpermissive temperature. Subsequently, phagocytotic activities were measured directly by incubating amoebae in shaken cultures in axenic medium using *E. coli* B/r, latex beads, and erythrocytes as particulate prey. Various particles have been employed to study the potential influence of different surface properties upon the acceptability of phagocytotic substrates. Mutants have been grouped into three classes according to their growth characteristics. Furthermore, mutants of each class could be divided into subclasses according to their phagocytotic properties (Table I).

CLASS I STRAINS: 15 mutant strains grow in axenic medium by pinocytosis at 20° and 27°C like wild-type cells with doubling times of ~8 h. 12 of these mutants (class I_A) phagocytose the particles used with initial rates comparable to those of wild-type cells at both temperatures, whereas three

TABLE I
Classification of Mutants* According to Their Growth Characteristics and Phagocytotic Properties in Axenic Medium

| Mutant class | No. mutants | Growth at 27°C | Phagocytosis‡ | |
|------------------------|-------------|----------------------------|---------------|------|
| | | | 20°C | 27°C |
| Class I _A | 12 | + | + | + |
| Class I _B § | 3 | (8 h doubling time) | - | - |
| Class II _A | 65 | - | + | - |
| Class II _B | 11 | (stop after 4-8 doublings) | - | - |
| Class III | 5 | - | + | - |
| (stop immediately) | | | | |

* All mutants are temperature-sensitive for growth in association with bacteria plated on nutrient agar and grow normally at 20°C in axenic medium.

‡ Phagocytosis was measured in axenic medium in shaken cultures using FITC-*E. coli* B/r, latex beads, and erythrocytes as substrate particles.

§ Haploid strains isolated in this laboratory are designated HV. The three strains of class I_B are denoted: HV29 (phg-1353, tsg-1353), HV32 (phg-1356, tsg-1356), HV33 (phg-1357, tsg-1357). Genes determining the phagocytosis phenotype are denoted by phg and those determining the temperature sensitivity for growth by tsg. The different numbers of mutant loci represent independently isolated mutations.

mutants (class I_B) do not phagocytose at all at any temperature when incubated in shaken cultures in axenic medium.

CLASS II STRAINS: Most of the mutants grow normally in axenic medium at 20°C. However, after shifting to 27°C they only grow initially, but growth decreases gradually and finally stops after four to eight generations. About half of these strains are irreversibly injured, whereas the others recover when shifted back to 20°C. Most of these mutants phagocytose normally at 20°C, but at 27°C the phagocytotic capacity decreases in parallel with the decreasing growth rate (class II_A). 11 strains were found that do not phagocytose in shaken cultures in axenic medium at all (class II_B).

CLASS III STRAINS: Five mutant strains are extremely temperature sensitive for growth in axenic medium and stop growing immediately after shifting to the restrictive temperature. Two of these mutants die at high temperature, whereas the others survive at least 2 d at high temperature and recover when shifted back to 20°C. These strains stop phagocytosis immediately after shifting to the higher temperature and recover again upon short time incubation (15 min) at 20°C.

In summary, most of the mutant strains temperature sensitive for growth via pinocytosis in axenic medium phagocytose normally at 20°C and the phagocytotic capacity decreases at 27°C in parallel with the decreasing growth rate (class II_A and class III). These strains could be impaired in any essential cellular process participating either directly or indirectly in endocytosis. 12 mutants (class I_A) grow and phagocytose normally in axenic medium at 20° and 27°C. Because these mutants are unable to grow on bacteria at the restrictive temperature, they are probably impaired in steps subsequent to uptake, for example, in digestion of bacteria. 14 mutants have been found that do not phagocytose in shaken cultures in axenic medium, either at the permissive or at the restrictive temperature. 11 of these strains belong to class II_B being in addition temperature sensitive for growth in axenic medium as well as on bacteria. This latter phenotype may be as a result of a second mutation. The extended treatment of amoebae with relatively high concentrations of the potent mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine heightens the incidence of multiple mutations.

The remaining three strains (class I_B), named HV29, HV32, and HV33, grow in axenic medium via pinocytosis at the permissive and restrictive temperature with wild-type characteristics but do not phagocytose any of the various substrate particles when incubated in axenic medium in agitated suspensions. These strains carry a mutation in phagocytotic uptake, designated as *phg*, without being impaired in other essential cellular functions. In these strains, development, fruiting body formation, and spore size are identical to the AX2 parent strain. Because these mutants exhibited a clear-cut phenotype which appeared to be directly related to the phagocytotic event, we decided to subject these isolates to further scrutiny.

Comparison of Endocytosis in Wild-type and Mutant HV32 Amoebae

Strains HV29, HV32, and HV33 have the same phenotype and, therefore, a detailed analysis of the endocytotic properties is presented for strain HV32 as a representative example. Pinocytosis was measured in axenic medium at 20° and 27°C. Consistently mutant amoebae have been found to pinocytose at about twice the rate of wild-type amoebae. Uptake rates are the same at both temperatures, and the results obtained at 20°C are presented in Fig. 2.

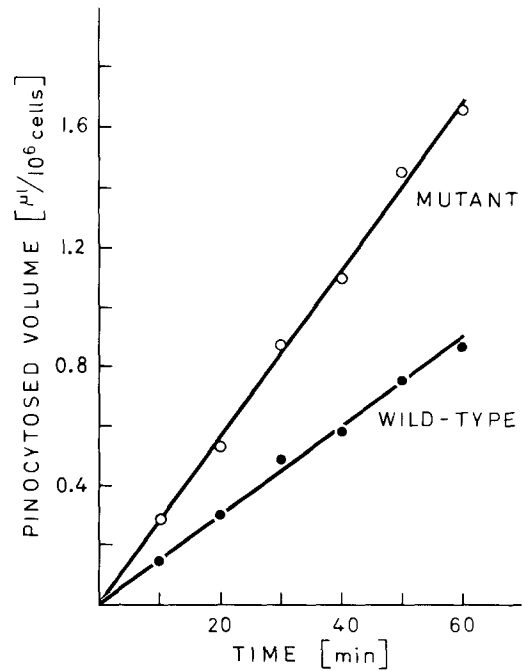


FIGURE 2 Comparison of pinocytotic activities of wild-type and mutant HV32 cells in axenic medium at 20°C.

Phagocytosis was initially measured by incubation of samples in axenic medium on a rotary shaker. Because high shear forces are generated by shaking, strong adhesion of substrate particles to the cell surface is a prerequisite for successful engulfment. Particles must remain bound to the cell surface long enough to permit enclosure within a phagocytotic vacuole. A wild-type amoeba under these conditions ingests about four *E. coli* B/r, eight latex beads, and 0.2 erythrocytes per min. Interestingly, in spite of the large differences in the sizes of the various particles, uptake rates in each case represent an internalization of roughly the same (25–35 μm^2) surface area per min. In contrast, mutant HV32 amoebae do not phagocytose any of these different particles as shown in Figs. 4, 5, and 7 for bacteria, latex beads, and erythrocytes, respectively.

To demonstrate that adhesion of substrate particles to HV32 amoebae in shaken cultures is actually the determining factor for internalization, phagocytosis was assayed in the absence of shear forces on filters. For this, amoebae were incubated with the various substrate particles on filters resting on pads saturated with axenic medium. Substrate particles are immobilized under these conditions and *E. coli* B/r, latex beads, and erythrocytes can be engulfed by mutant and wild-type amoebae with comparable rates at 20° and 27°C (cf. Fig. 3). Apparently, an initial binding step is affected by mutation in mutant HV32 cells, leading to the inability of phagocytosis in agitated suspensions.

When agitated in phosphate buffer with *E. coli* B/r as the sole food source, mutant and wild-type amoebae grow with the same doubling time of ~ 2.5 h at 20°C.

Because growth is dependent upon phagocytosis under these conditions, inhibition of phagocytosis appears to be caused by components contained in axenic medium. Axenic medium contains glucose, peptone, and yeast extract in phosphate buffer. Consequently, phagocytosis of the various substrate particles in shaken cultures was measured in phosphate buffer in the presence of each of these components.

Uptake of *E. coli* B/r by mutant HV32 cells is completely inhibited by glucose but proceeds with high rates in wild-type

amoebae (Fig. 4). Mutant and wild-type amoebae ingest bacteria with similar rates in buffer alone and after addition of peptone or yeast extract. In contradiction, uptake of latex beads by mutant HV32 cells is completely inhibited in the presence of peptone and yeast extract but in phosphate, or in the presence of glucose, uptake proceeds normally as compared to wild-type cells (Fig. 5). Uptake of *E. coli* B/r and latex beads was also measured simultaneously in the same batch of mutant cells in phosphate buffer alone or in the presence of glucose or peptone (Fig. 6). Glucose selectively inhibits the uptake of bacteria, whereas ingestion of latex beads is not affected. On the other hand, only uptake of latex beads is blocked in the presence of peptone, while ingestion of bacteria remains rapid.

Uptake of erythrocytes could only be determined quantitatively in axenic medium (Fig. 7) but not in 20 mM phosphate buffer, as erythrocytes lyse in hypotonic medium. On the other hand, amoebae ingest poorly in high salt solutions such as physiological saline. Erythrocytes were stabilized by fixation with glutaraldehyde, and phagocytosis was determined qualitatively by microscope observation after incubation with amoebae

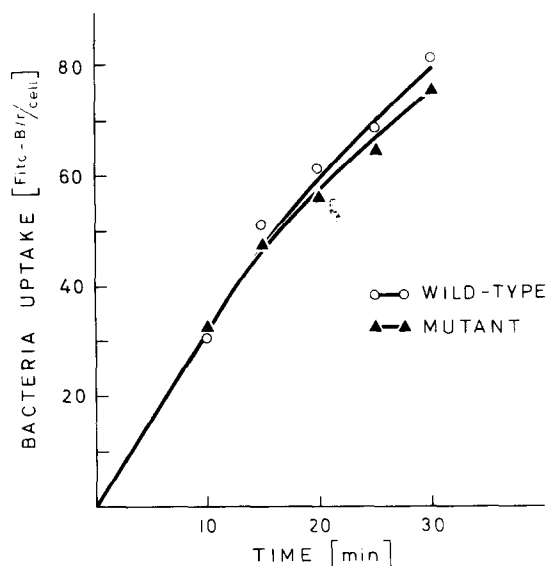


FIGURE 3 Comparison of *E. coli* B/r uptake by wild-type and mutant HV32 cells after incubation at 20°C on filters resting on support pads presoaked with axenic medium.

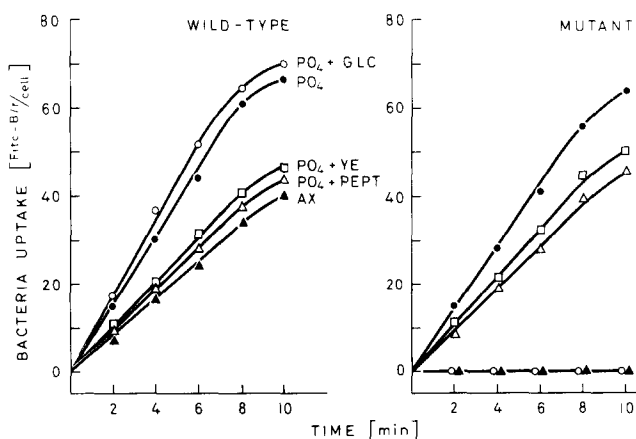


FIGURE 4 Comparison of *E. coli* B/r uptake at 20°C by wild-type and mutant HV32 amoebae in shaken cultures in axenic medium (▲), in 20 mM phosphate buffer, pH 6.2 (●), and in phosphate buffer supplemented with 50 mM glucose (○), 10 mg/ml bacto-peptone (△), 10 mg/ml yeast extract (□).

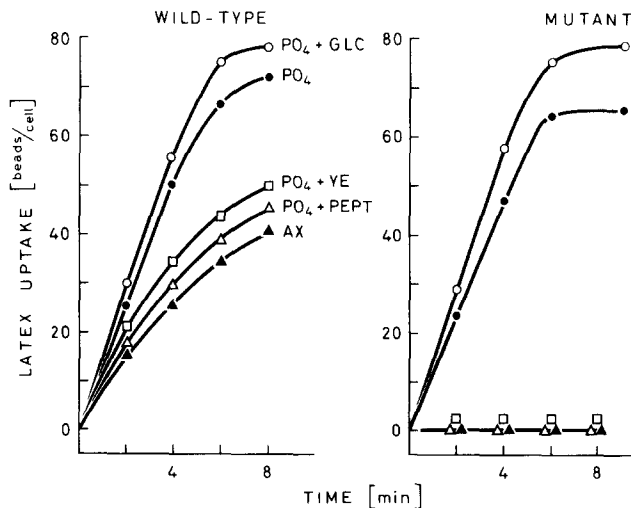


FIGURE 5 Comparison of latex uptake at 20°C by wild-type and mutant HV32 amoebae in shaken cultures in axenic medium (▲), in 20 mM phosphate buffer, pH 6.2 (●), and in phosphate buffer supplemented with 50 mM glucose (○), 10 mg/ml bacto-peptone (△), 10 mg/ml yeast extract (□).

bae in phosphate buffer on a rotary shaker. Only wild-type cells were observed to ingest fixed erythrocytes under these conditions, whereas mutant HV32 cells could not internalize erythrocytes, even in phosphate buffer.

To summarize, wild-type cells appear to be indiscriminate regarding the nature of substrate particles taken up. However, mutant HV32 cells disclose clear-cut preferences for the type of particles. Uptake of *E. coli* B/r is selectively inhibited by glucose, whereas latex uptake is selectively inhibited by components contained in peptone or yeast extract. Finally, mutant HV32 amoebae are not capable of ingesting erythrocytes at all. We may conclude from these observations that functionally independent recognition or binding sites are present on the cell surface of *D. discoideum* and that binding properties of mutant HV32 amoebae are altered by mutation.

Specificity and Mode of Inhibition of Phagocytosis by Sugars and Peptone in Mutant HV32

Uptake rates for *E. coli* B/r were measured in the presence of various sugars and the results are listed in Table II. All glucose derivatives with different anomeric configuration or different substitution on the C1-carbon of glucose are strong inhibitors (group *a*). However, rather strict structural requirements for inhibition are found at other positions in the sugar. Derivates of glucose such as deoxyglucose or *N*-acetylglucosamine (cf. group *b*), or diastereomeric sugars such as mannose, allose, and galactose (cf. group *c*) are far less effective. When oligosaccharides are used as inhibitors, glucose has to be bound glycosidically at the terminus. Lactose (Gal-β-1,4-Glc) for instance is only a poor inhibitor. Phagocytosis of bacteria by wild-type amoebae is not significantly influenced by the sugars listed above.

The type of inhibition of glucose for *E. coli* B/r uptake was analyzed in analogy with respect to enzyme kinetics. Phagocytosis was measured with subsaturating amounts of bacteria in the presence of various concentrations of glucose. After plotting of reciprocal uptake rates against reciprocal concentrations of bacteria, straight lines of differing slope with a common intercept on the ordinate were obtained (Fig. 8). This indicates

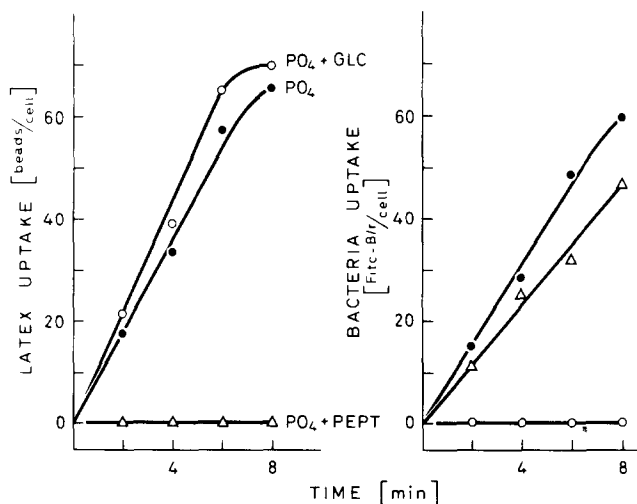


FIGURE 6 Uptake of latex and *E. coli* B/r at 20°C measured simultaneously in the same bath of mutant HV32 cells in shaken cultures in 20 mM phosphate, pH 6.2 (●), and in phosphate buffer supplemented with 20 mM glucose (○) and 1 mg/ml bactopectone (Δ).

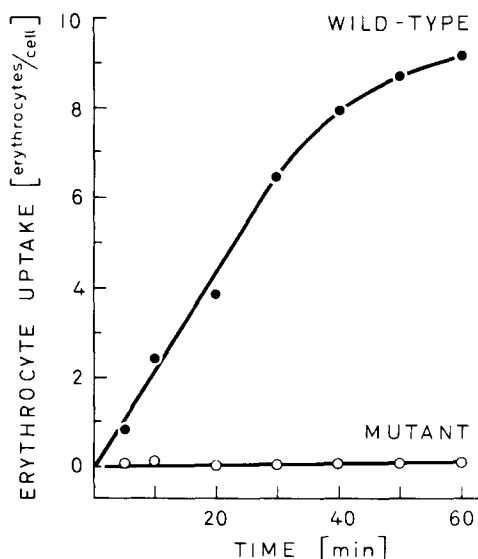


FIGURE 7 Comparison of erythrocyte uptake at 20°C by wild-type and mutant HV32 amoebae in shaken cultures in axenic medium.

that glucose is a competitive inhibitor of *E. coli* B/r uptake, and the apparent inhibition constant was found to be ~0.7 mM.

E. coli B/r contains glycosidically linked terminal glucose residues (14). Glucose inhibits specifically and competitively the uptake of these bacteria by mutant HV32 cells. These findings strongly suggest that reversible binding of bacteria to amoebae is achieved by a glucose-binding protein. To test this possibility further, a lipopolysaccharide mutant of *E. coli* (K2754) which does not contain terminal glucose residues on the surface (15), was chosen as a phagocytotic substrate. In phosphate buffer, wild-type and mutant amoebae ingest the parent K-12 *E. coli* cells, containing terminal glucose residues, at rates very similar to those of *E. coli* B/r cells and the uptake is inhibited by glucose in mutant amoebae (data not shown). The nonglycosylated K2754 bacteria are ingested by wild-type amoebae at rates comparable to those of the glucose-containing *E. coli* B/r (Fig. 9). In contrast, mutant amoebae cannot phagocytose the glucose-free bacteria under any conditions.

Taken together, these observations can be plausibly explained by the assumption that wild-type cells contain a glucose-binding protein and an additional binding site that is altered by mutation in mutant HV32 cells.

A clear-cut analysis of inhibition of latex uptake by peptone or yeast extract in mutant cells seemed to be difficult because peptone and yeast extract are complex mixtures chemically not well defined. Peptone was found to be effective as inhibitor at concentrations as low as 10–20 μg/ml, whereas ~0.5–1 mg/ml of yeast extract was necessary for complete inhibition. Polystyrene latex spheres are very hydrophobic and many proteins such as immunoglobulins are tightly bound to the surface of

TABLE II
Sugar Specificity for Inhibition of *E. coli* B/r Uptake in Mutant HV32 Amoebae

- | |
|---|
| a. Strong inhibitors* (100% inhibition) |
| D-glucose |
| Maltose (Glc-α-1,4-Glc) |
| Cellobiose (Glc-β-1,4-Glc) |
| Sucrose (Glc-α-1,2-Glc) |
| 1-thio-D-glucose |
| D-glucose-L-cystein |
| b. Moderate inhibitors (~30–40% inhibition) |
| 6-Deoxyglucose |
| 2-Deoxyglucose |
| Glucosamine |
| N-acetylglucosamine |
| c. Weak inhibitors (almost no inhibition) |
| Mannose |
| Allose |
| Galactose |
| Lactose |

* Phagocytosis was measured in the presence of 20 mM of the appropriate saccharide dissolved in phosphate buffer (20 mM, pH 6.2) and compared to uptake rates obtained in the absence of saccharide. Phagocytosis of bacteria in wild-type cells is not significantly influenced in the presence of the sugars listed above.

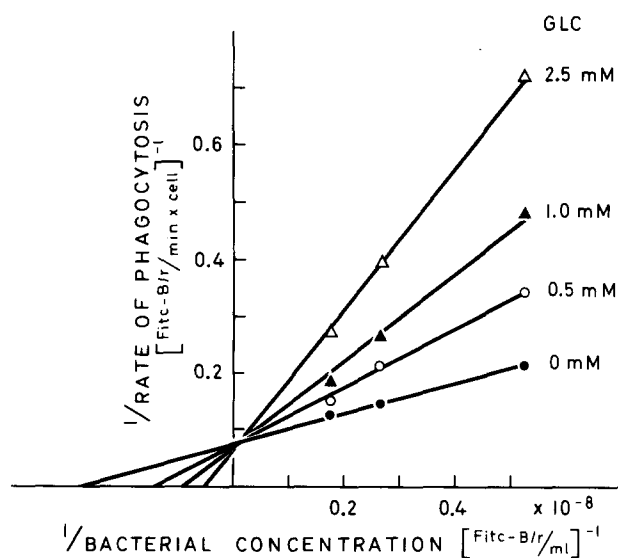


FIGURE 8 Competitive inhibition of *E. coli* B/r uptake in mutant HV32 cells by glucose. Phagocytosis was measured in 20 mM phosphate, pH 6.2, at 20°C after incubation of cells with different amounts of bacteria in the presence of various concentrations of glucose as indicated. Reciprocal rates of phagocytosis were plotted against reciprocal values of bacteria concentrations.

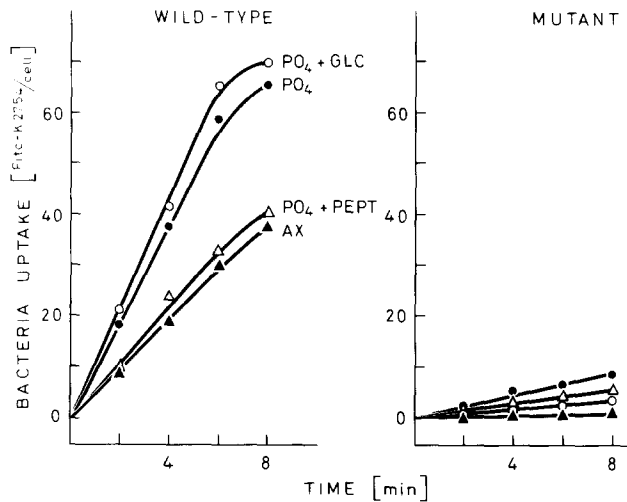


FIGURE 9 Comparison of *E. coli* K2754 (heptose-minus) uptake at 20°C by wild-type and HV32 amoebae in shaken cultures in axenic medium (\blacktriangle), in 20 mM phosphate buffer, pH 6.2 (\bullet), and in phosphate buffer supplemented with 50 mM glucose (\circ), 10 mg/ml bacto-peptone (Δ). (The heptose-less lipopolysaccharide-mutant strain *E. coli* K2754 was isolated by Dr. K. Jann and kindly given by Dr. U. Henning.)

latex beads (16). Because peptone, a tryptic digest of meat, contains high amounts of amino acids and oligopeptides, the possibility was investigated that components from peptone were bound to latex beads and thereby change their surface properties in a way that binding to mutant amoebae is prevented. Latex beads were preincubated in a peptone solution (10 mg/ml) and washed with phosphate buffer. Subsequently, phagocytotic uptake by wild-type and mutant HV32 amoebae was determined in phosphate buffer. The pretreated latex beads were no longer ingested by mutant HV32 cells, but uptake proceeds normally in wild-type amoebae (Fig. 10). This effect is not specific for a certain component of peptone, because latex beads coated with FITC-labeled anti-rabbit immunoglobulin (Fig. 10) or serum albumin (data not shown) were not phagocytosed either. The coating of the latex bead by fluorescein-conjugated immunoglobulin was confirmed by fluorimetric examination. Therefore, inhibition of latex uptake by peptone is not specific but seems to be caused by different surface properties of latex beads after coating with proteins or peptides. An explanation for this observation could be that coating with protein renders the hydrophobic polystyrene spheres more hydrophilic. Wild-type amoebae apparently do not discriminate between strong hydrophobic and more hydrophilic particles, but ingest both equally well. However, mutant HV32 cells seem to be altered in such a way that successful interaction is achieved only with hydrophobic particles. Consequently, the protein-coated and therefore more hydrophilic latex beads can not be phagocytosed.

Cohesiveness of Mutant HV32 Cells Compared to Wild-type Cells

Exponentially growing wild-type cells cohere rapidly when resuspended in phosphate buffer. Within 15 min, almost all cells form large, tight aggregates as revealed by microscope observation (Fig. 11). During early development, this kind of aggregation is inhibited or reversed by EDTA (17). In contrast to wild-type cells, almost all mutant HV32 cells remained as

single cells after identical pretreatment (Fig. 11). During acquisition of aggregation competence, EDTA-resistant cohesiveness develops in wild-type cells (17). This is also the case for mutant cells. They start to form tight aggregates after 8–10 h of incubation in phosphate buffer, which are resistant to EDTA treatment. Furthermore, adhesion to foreign surfaces is also altered in mutant cells. Wild-type cells suspended in axenic medium or in phosphate buffer and incubated without shaking in polystyrene petri dishes adhere tightly to the surface in both media. In contrast, mutant cells adhere only when incubated in phosphate buffer, but remain in suspension when incubated

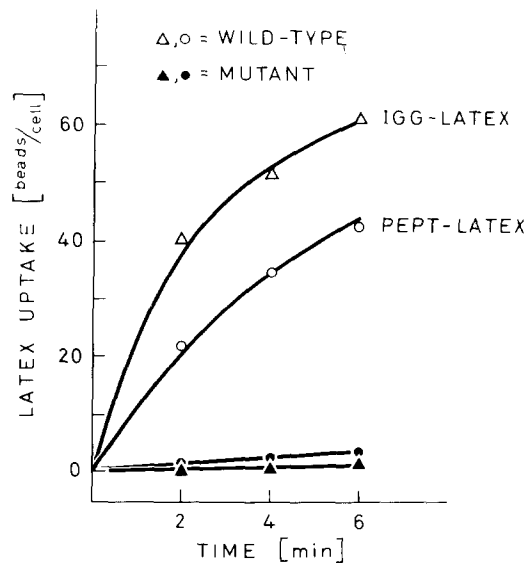


FIGURE 10 Uptake of latex beads precoated with bacto-peptone components (\circ , \bullet) or anti-rabbit FITC-immunoglobulin G (Δ , \blacktriangle) by wild-type and mutant HV32 cells in shaken cultures in 20 mM phosphate, pH 6.2, at 20°C.

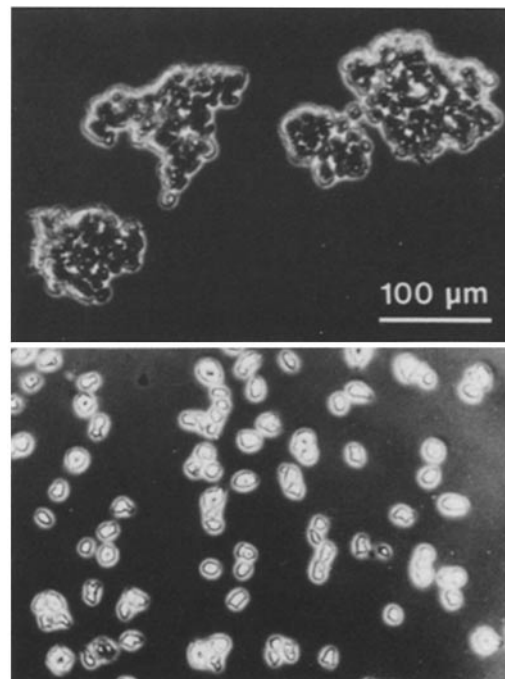


FIGURE 11 Cell aggregation of wild-type (top) and mutant HV32 (bottom) amoebae after incubation of axenically grown cells for 15 min in 20 mM phosphate, pH 6.2, at 20°C. $\times 100$.

in axenic medium. This behavior parallels the binding properties of mutant cells for hydrophobic polystyrene latex particles and for more hydrophilic protein-coated latex particles. The observation suggests that adherence of cells to an extended surface reflects their attempt to phagocytose a particle of infinite size.

Isolation of Temperature-insensitive Revertants

Mutant strains HV29, HV32, and HV33 have an identical phenotype according to the criteria described above. No direct correlation was detected between altered phagocytotic properties in these strains and their temperature sensitivity for growth on bacteria plated on agar. We have isolated spontaneous revertants that have regained the ability to grow on bacterial plates at 27°C. Revertants arose with a frequency of about 2×10^{-5} for strains HV29 and HV32 and with a frequency of about 5×10^{-7} for strain HV33. Four revertants of each strain were characterized more closely, and all of the revertants displayed the mutant phenotype for phagocytosis and cohesiveness. Therefore, temperature sensitivity is caused by a secondary mutation which is unrelated to the mutation causing the altered phagocytotic phenotype.

DISCUSSION

The major finding of the present work was the identification of two alternative mechanisms for recognition in the phagocytotic process of the unicellular slime mold *D. discoideum*. This was achieved by isolation of mutants with altered phagocytotic properties. Temperature-sensitive phagocytosis mutants have been described previously (18). However, up to the present time these mutations could not be attributed unambiguously to the endocytotic process because the impairment of other essential cellular activities could not be excluded. We have found three mutant strains exhibiting a phenotype which is unequivocally related to the process of phagocytosis per se. Analysis of the mutant phenotype revealed that functionally independent binding sites are present on the cell surface of *D. discoideum* which recognize different surface properties on a particulate prey. Based on the data presented above, the following conclusions can be drawn (cf. Fig. 12):

First, polystyrene latex beads, having a very hydrophobic surface (19), are bound and internalized by wild-type and mutant amoebae equally well. Latex beads do not carry functional groups that can be imagined to interact specifically with a cell surface component. Thus, mere physical forces seem to promote adhesion between cells and latex particles. High interfacial tension between the particles and the surrounding medium, but low interfacial tension against the phagocytotic cell favors binding and phagocytosis (19, 20). Because the existence of specific membrane receptors for these particles is unlikely, the term "nonspecific receptor" has been used to characterize cell surface components mediating this type of binding (2). Based on kinetic data, a saturable population of "binding sites" for latex beads has also been suggested to exist on the cell surface of *Acanthamoeba* (21). *D. dictyostelium* wild-type amoebae appear to internalize a wide variety of substrate particles with relatively hydrophilic (e.g., bacteria, erythrocytes) and strongly hydrophobic (latex) surface properties after binding to this nonspecific receptor. The phg mutations in strains HV29, HV32, and HV33 apparently have altered the surface properties of these cells in such a way that their ability for nonspecific binding by hydrophobic interaction is changed.

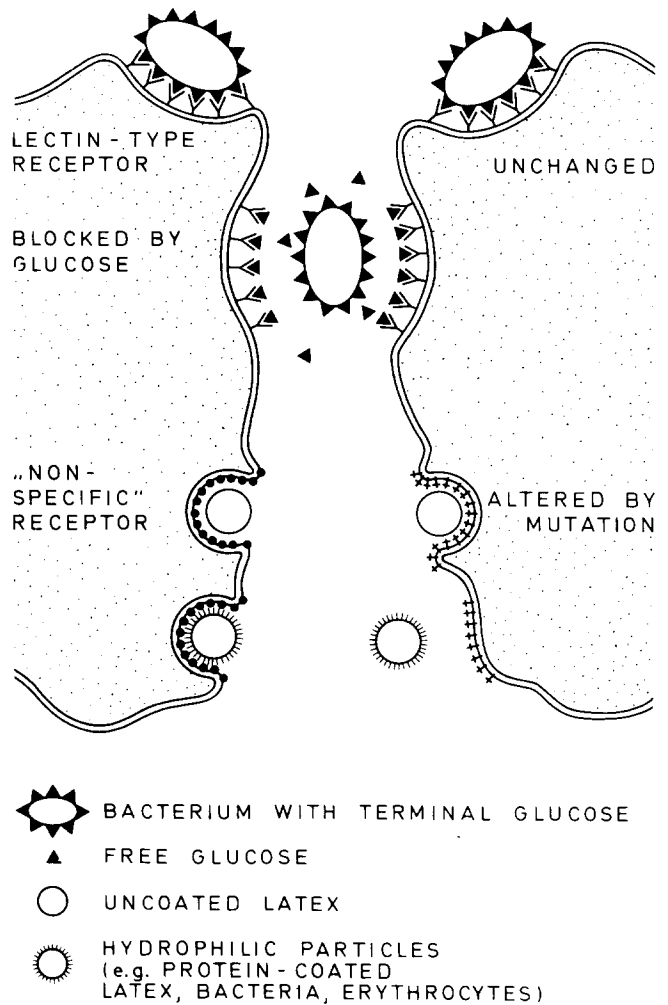


FIGURE 12 Model depicting the presence of different receptors for phagocytosis on the cell surface of *D. discoideum*.

Only the strongly hydrophobic polystyrene latex beads can still be bound and ingested by this receptor but not more hydrophilic particles such as protein-coated latex beads, bacteria, and erythrocytes.

Second, characterization of the mutant phenotype with respect to phagocytosis disclosed another binding site. Mutant cells avidly ingest *E. coli* B/r, a bacterium containing terminal glucose residues in a glycosidic linkage (14). Uptake of these bacteria is inhibited specifically by glucose and by oligosaccharides containing glycosidically linked terminal glucose residues. The inhibition is competitive with an inhibition constant of ~ 0.7 mM. This finding strongly suggests that binding of these bacteria is a specific, reversible carbohydrate recognition. The recognition site might be a monovalent or multivalent lectinlike protein. The existence of this binding site is overshadowed in wild-type amoebae, as bacteria can also be ingested by the nonspecific recognition mechanism.

Strong evidence in favor of this model is the observation that *E. coli* cells without glucose residues on the cell surface (*E. coli* K2754) cannot be phagocytosed by mutant amoebae under any conditions, whereas wild-type amoebae ingest these bacteria at rates comparable to that of the glucose-containing *E. coli* B/r (cf. Fig. 9). The lectin-type receptor cannot recognize *E. coli* K2754, because glucose is not present on the cell surface. Wild-type amoebae can ingest these bacteria via the nonspecific receptor, but this recognition site is altered in mutant amoebae

TABLE III

Interpretation of the Observed Phenotypic Behavior of Wild-type and Mutant HV29, HV32, and HV33 Amoebae in Terms of a Model Considering Independent Recognition Sites for Phagocytosis

| Substrate particles and incubation conditions | Binding via lectin-receptor | | Binding via nonspecific receptor | | Phenotype observed | |
|--|-----------------------------|--------------------------|----------------------------------|---|--------------------|--------------------------|
| | Wild-type AX2 | Mutants HV29, HV32, HV33 | Wild-type AX2 | Altered in mutant cells Mutants HV29, HV32, HV33 | Wild-type AX2 | Mutants HV29, HV32, HV33 |
| <i>E. coli</i> B/r (terminal glucose, glucose-free medium) | + | + | + | - | + | + |
| <i>E. coli</i> B/r (glucose present in the medium) | - | - | + | - | + | - |
| <i>E. coli</i> K2754 (glucoseless) | - | - | + | - | + | - |
| Latex beads | - | - | + | + | + | + |
| Latex beads coated with proteins | - | - | + | - | + | - |
| Erythrocytes | - | - | + | - | + | - |

and will not interact with the relatively hydrophilic surface of bacteria. Therefore, ingestion of these bacteria by mutant cells cannot be achieved by either recognition mechanism. Furthermore, it can be concluded from this experiment that the lectin-type receptor is located on the amoeba cell surface and not on the bacterial surface. This situation is just opposite to that observed for phagocytosis of *E. coli* by mouse peritoneal phagocytes (22). In this case, mannose residues on the phagocyte surface seen to be recognized by a bacterial lectin.

In Table III the phagocytotic properties of wild-type and mutant amoebae are summarized. The observed mutant phenotype regarding different substrate particles and incubation conditions agrees perfectly well with the behavior predicted on the basis of the proposed model.

All three mutants with altered properties in cell-particle binding in phagocytosis are, in addition, altered in other cohesive properties of the cells. When suspended in axenic medium, they do not adhere to plastic surfaces and mutant cells do not form EDTA-sensitive aggregates, when suspended in phosphate buffer. Because the mutants are of independent origin it seems likely that there is a common basis for these properties. Unspecific cohesiveness of the cells is probably determined by interfacial tension which itself is determined by the hydrophobicity of the cell surface (19). If the mutations described here cause more hydrophilic surface properties of mutant cells compared to wild-type cells, a general change of cohesive properties as observed here could result. It is probably this change in cohesive properties which led to enrichment of these cells in the selection procedure. Cells that do not phagocytose in axenic medium and, in addition, have no tendency to clump together are expected to be selectively enriched in the supernate after tungsten treatment. Nevertheless, these mutants would have been lost during the subsequent screening had they not carried an accidental second but unrelated mutation that conferred a temperature-sensitive phenotype. In *D. discoideum* two functionally independent mechanisms for cell aggregation have been identified (17). EDTA-sensitive side-by-side cohesion of vegetative cells is mediated by contact sides B, whereas EDTA-resistant end-to-end cohesion of aggregation-competent cells is mediated by contact sides A. Different glycoproteins (23, 24) and carbohydrate-binding proteins seem to be necessary for proper development (25-27). Mutants HV29, HV32, and HV33 do not form EDTA-sensitive aggregates. Therefore,

contact site B-mediated side-by-side association seems to be impaired in these cells. Because the mutants develop normally after acquisition of aggregation competence, contact site B-mediated cohesion is not necessary for proper development, but seems to be involved only in physical attraction between the cell surface and a second surface. This might be another amoeba cell, a substrate particle, or an extended glass or plastic surface. Little is known chemically about membrane components which determine the "stickiness" of cell surfaces. Biochemical comparison of wild-type and mutant amoebae will possibly allow the identification of these components.

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