

Enteropathogenic *Escherichia coli* Inhibits Phagocytosis

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Enteropathogenic *Escherichia coli* (EPEC) interacts with intestinal epithelial cells, activating host signaling pathways leading to cytoskeletal rearrangements and ultimately diarrhea. In this study, we demonstrate that EPEC interacts with the macrophage-like cell line J774A.1 to inhibit phagocytosis by these cells. Antiphagocytic activity was also observed in cultured RAW macrophage-like cells upon EPEC infection. The EPEC antiphagocytic phenotype was dependent on the type III secretion pathway of EPEC and its secreted proteins, including EspA, EspB, and EspD. Intimin and Tir mutants displayed intermediate antiphagocytic activity, suggesting that intimate attachment mediated by intimin-Tir binding may also play a role in antiphagocytosis. Tyrosine dephosphorylation of several host proteins was observed following infection with secretion-competent EPEC but not with secretion-deficient mutants. Dephosphorylation was detectable 120 min after infection with EPEC, directly correlating with the onset of the antiphagocytic phenotype. Inhibition of protein tyrosine phosphatases by pervanadate treatment increased the number of intracellular wild-type EPEC organisms to levels seen with secretion-deficient mutants, suggesting that dephosphorylation events are linked to the antiphagocytic phenotype. No tyrosine phosphatase activity was detected with the EPEC-secreted proteins, suggesting that EPEC induces antiphagocytosis via a different mechanism than *Yersinia* species. Taken together, the present findings demonstrate a novel function for EPEC-secreted proteins in triggering macrophage protein tyrosine dephosphorylation and inhibition of phagocytosis.

Enteropathogenic *Escherichia coli* (EPEC), a human pathogen, is a leading cause of infantile diarrhea in developing countries, killing several hundred thousand children per year worldwide (21). Despite the significance of this disease, the mechanisms by which EPEC causes diarrhea remain poorly characterized. It has been demonstrated both in vitro and in vivo that EPEC organisms initially adhere nonintimately in microcolonies to host intestinal epithelial cells via bundle-forming pili (Bfp) (13). Upon initial adherence, several bacterial proteins are secreted by a type III secretion pathway, of which at least three, EspA, EspB, and EspD, are involved in activating host signals (10, 14–16, 20). Induction of host signaling events leads to rearrangement of the host cytoskeleton to form the characteristic attaching and effacing lesion, resembling a pedestal structure upon which the organism resides (25). The signaling events include induction of host inositol triphosphate and Ca²⁺ fluxes, as well as host protein phosphorylation (3, 8, 9, 16, 24). Tir is a bacterially secreted protein which is inserted into host cell membranes and tyrosine phosphorylated (18). Binding of Tir to the EPEC outer membrane protein intimin leads to intimate binding of the pathogen to the host cell and pedestal formation (25, 18).

Tyrosine dephosphorylation of host proteins also occurs within the host epithelial cell following EPEC infection, but its role in the attachment process remains undefined (16). This dephosphorylation occurs independently of intimin. Furthermore, inhibition of tyrosine dephosphorylation does not prevent pedestal formation, suggesting that it may play another

role in the infection process (16). Pathogenic *Yersinia* species induce tyrosine dephosphorylation of macrophage proteins to paralyze host phagocytic activity (26, 27). This event requires a bacterial tyrosine phosphatase, YopH, which is translocated into the host cell (4, 2).

The rabbit form of EPEC, RDEC-1, inhibits its uptake by membranous (M) cells on intestinal surfaces, but the mechanism mediating antiphagocytosis remains undefined (12). This pathogen is very similar to EPEC, secreting EspA, EspB, and Tir and producing attaching and effacing lesions (1). M cells are specialized epithelial cells which engulf many bacteria (except RDEC-1), bacterial components, and other antigens from the lumen transporting them into contact with the underlying antigen-presenting cells of the gut-associated lymphoid tissue (GALT) (30). An antiphagocytic phenotype may be advantageous in that it allows the bacteria to colonize the intestinal lining and M cells without being transported to the GALT, thereby delaying an elicited immune response.

Given the antiinvasive phenotype of RDEC-1 and the occurrence of tyrosine dephosphorylation events upon EPEC infection of epithelial cells, we investigated the possible relationship between EPEC-induced dephosphorylation and antiphagocytic activity.

MATERIALS AND METHODS

Cell culture and bacterial growth. The mouse phagocytic cell lines J774A.1 (22) and RAW (23) were grown in Dulbecco's minimal Eagle medium (DMEM) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO₂. EPEC and *Yersinia pseudotuberculosis* strains used in this study are listed in Table 1. EPEC strains were cultured in Luria-Bertani broth overnight at 37°C without shaking. *Y. pseudotuberculosis* strains were grown in brain-heart infusion broth at 26°C overnight on a rotary shaker. These cultures were diluted to 10⁸ bacteria/ml (optical density at 550 nm, 0.1) and further incubated at 26°C for 1 h and then at 37°C for 2 h prior to infection of J774 cells.

Determination of bacterial uptake by J774 cells by immunofluorescence. Two days prior to infection, J774 cells were seeded onto coverslips (11-mm diameter; 5 × 10⁴ cells/well) in a 24-well microtiter plate. Monolayers were routinely infected with EPEC (multiplicity of infection, 50) for 180 min (except for time course experiments). Bacterial uptake was stopped by placing the microtiter

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description	Reference
Strains		
EPEC		
E2348/69	Wild type	6
UMD864	E2348/69 with <i>espB</i> deletion	11
UMD872	E2348/69 with <i>espA</i> deletion	15
UMD870	E2348/69 with <i>espD</i> deletion	20
CVD206	E2348/69 with <i>eaeA</i> deletion	7
E2348/69 Δ <i>tiz</i>	E2348/69 with <i>tiz</i> deletion	18
cfm 14-2-1(1)	<i>cfm::TnPhoA</i>	6
31-6-1	<i>bfpA::TnPhoA</i>	13
<i>Y. pseudotuberculosis</i>		
YPIII(pIB102)	Wild type	5
YPIII(p ⁻)	Plasmid cured	5
Plasmid		
pCVD450	Encodes the <i>per</i> regulon	11

plates on ice. Intra- and extracellular bacteria were determined as described previously for *Y. pseudotuberculosis* (2, 26). Briefly, to stain extracellular bacteria, coverslips were washed and incubated with rabbit anti-EPEC antibodies (1:100 dilution) (11) and incubated for 30 min at 4°C. Monolayers were washed four times with phosphate-buffered saline (PBS) and fixed with ice-cold methanol for 90 s. After methanol removal, extracellular bacteria were labeled with Texas red-conjugated goat anti-rabbit antiserum (1:200 dilution; Jackson Laboratories) for 20 min at 37°C, followed by four washes in PBS. Both intra- and extracellular bacteria were then stained by incubation of coverslips for 1 h at 37°C with anti-EPEC antisera and were washed four times in PBS. Coverslips were then incubated for 20 min at 37°C with fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (1:200 dilution; Jackson Laboratories). After four washes in PBS, coverslips were mounted in Mowiol mounting medium on a glass slide. Extracellular bacteria were detected by excitation at 596 nm, and total cell-associated bacteria were detected at 490 nm. For each experiment, 50 cells per coverslip with 10 to 20 cell-associated bacteria were randomly selected, and the numbers of extracellular and total cell-associated bacteria were determined. To determine the effects of bacterial numbers on antiphagocytosis, 50 cells per coverslip were counted with either 0 to 5, 5 to 15, or 15 to 50 bacteria per cell.

Preparation of cell lysates for Western blotting. J774 monolayers were cultured to confluency in 60-mm plates and infected with an overnight EPEC culture for 3 h (multiplicity of infection, 50) in serum-free DMEM. The monolayers were washed three times in PBS and resuspended directly in boiling Laemmli sample buffer (19). Protein samples were resolved by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS-PAGE) (19), and the proteins were transferred to nitrocellulose as described elsewhere (29). Blots were blocked in 4% bovine serum albumin BSA in PBS prior to incubation with antiphosphotyrosine (4G10 [1:2,000 dilution]; Upstate Biotechnology Inc.), and phosphotyrosine proteins were detected by alkaline phosphatase-conjugated secondary antibodies, as described previously (24).

Cell viability assays. Trypan blue (0.08% final volume; Gibco) was added to wells containing infected monolayers of J774 cells (200 μ l/ml of DMEM) for 10 min, followed by four washes in PBS. Cells were visualized by phase-contrast microscopy and the percentage of blue cells was determined.

Inhibition of protein tyrosine phosphatases by pervanadate. Hydrogen peroxide (2 mM) was added to 0.1 mM vanadate (VO₄; Sigma) to make pervanadate in DMEM. This solution was added to infected J774 cells following 90 min of EPEC infection, and cells were incubated for an additional 60 min. Control experiments with untreated cells or cells treated with H₂O₂ or VO₄ only were performed in parallel. After treatment, cells were prepared for fluorescence microscopy or for Western analysis as described above.

Detection of protein tyrosine phosphatase activity. To detect phosphatase activity of EPEC-secreted proteins and of *Y. pseudotuberculosis* Yop proteins in vitro, a tyrosine phosphatase assay kit (Boehringer Mannheim) was used according to the manufacturer's instructions. EPEC was grown under optimal conditions for secretion of Esp proteins, as described elsewhere (17). Briefly, EPEC organisms transformed with plasmid CVD450 were grown standing overnight at 37°C in Luria-Bertani broth supplemented with tetracycline (25 μ g/ml, final concentration). The culture was then diluted 1:100 in M9 medium supplemented with 0.45% glucose, 0.2% Casamino Acids, and 0.4% (wt/vol) NaHCO₃. EPEC cultures were grown to an optical density at 600 nm of 0.6 and centrifuged (16,000 RCF) for 2 min to pellet the bacteria. M9 supernatant containing the secreted proteins was concentrated with a Centricon-10 (Amicon). To collect *Y. pseudotuberculosis* Yop proteins, the bacteria were grown as described above and concentrated in the Centricon-10.

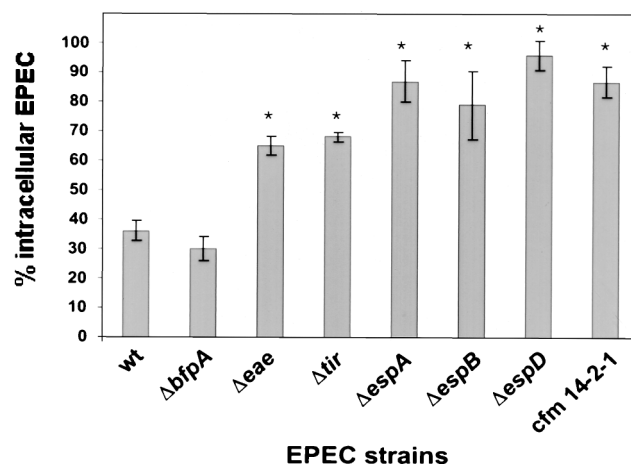


FIG. 1. EPEC inhibits its own uptake by J774 cells. J774 monolayers were infected with various EPEC strains for 3 h and prepared for fluorescence microscopy as described in Materials and Methods. Data are averages and standard errors of the means for three independent experiments performed in duplicate. *, significant difference from the wild type (wt) at P of <0.05 .

RESULTS

Phagocytosis of EPEC by J774 cells. To examine whether EPEC exhibited any antiphagocytic activity, cultured J774 cells were infected with wild-type EPEC and a type III secretion-deficient mutant, *cfm*, followed by immunofluorescence to determine the numbers of extracellular and total cell-associated bacteria. Wild-type EPEC inhibited its own uptake into J774 cells (only 36% of cell-associated bacteria were intracellular), whereas *cfm* was internalized in significantly greater numbers (87% intracellular [$P < 0.01$]) (Fig. 1). Similar results were obtained with RAW cells, with 19% of wild-type EPEC being intracellular compared to 66% of the *cfm* strain being internalized. To determine the potential roles of different EPEC gene products in mediating antiphagocytosis, several EPEC mutants were examined by immunofluorescence. The Bfp mutant inhibited its own uptake into J774 cells at levels comparable to that seen by wild-type EPEC (30% intracellular). In contrast, the type III secretion- and signaling-defective Δ *espA*, Δ *espB*, and Δ *espD* mutants were internalized in significantly greater numbers than the parental strain ($P < 0.01$), with 87, 79, and 96% of cell-associated bacteria found inside the cell, respectively. The Tir and intimin (*eae*) mutants showed intermediate phenotypes, with 65 and 68% intracellular bacteria, respectively.

It is possible that the lack of intracellular EPEC could be due simply to EPEC inducing J774 cell death and not preventing uptake at all. To examine this possibility, EPEC-infected macrophages were assayed for membrane integrity by trypan blue exclusion. No significant J774 membrane permeability was observed during the 3-h infection period with any of the EPEC strains.

Kinetics of EPEC-induced antiphagocytosis. To further characterize the antiphagocytic effect of EPEC on J774 cells, the numbers of cell-associated EPEC (wild type and *cfm*) and the percentages of those that were intracellular were determined at 60, 120, and 180 min postinfection (Fig. 2A and B). EPEC exhibited increasing adherence and antiphagocytosis (i.e., decreasing numbers of intracellular bacteria) with increasing time. At 60 min after bacterial addition, J774 cells had approximately five EPEC associated per cell, 78% of which were intracellular. At 120 and 180 min after bacterial addition,

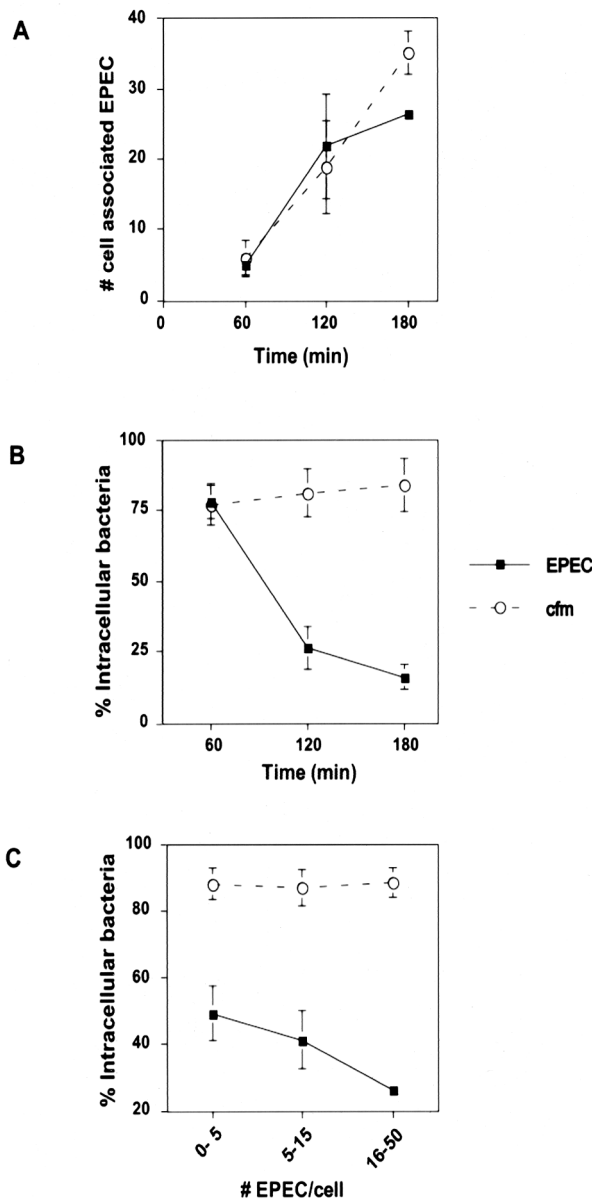


FIG. 2. EPEC-mediated antiphagocytosis is evident after 120 min of infection of J774 cells and correlates with increasing numbers of cell-associated bacteria. J774 cells were infected with EPEC or *cfm* for 60, 120, or 180 min. Monolayers were prepared for fluorescence microscopy as described in Materials and Methods. (A) Numbers of total cell-associated bacteria determined as a function of time. (B) Percentages of intracellular cell-associated bacteria determined at 60, 120, and 180 min of infection. (C) Percentages of intracellular bacteria determined for cells with 0 to 5, 5 to 15, or 15 to 50 associated bacteria per cell at 180 min after bacterial addition to macrophages. Data are averages and standard errors of the means for three independent experiments performed in duplicate.

EPEC adherence to J774 cells increased significantly relative to 60 min of infection ($P < 0.05$). Correlated with the increased adherence was a significant increase in antiphagocytic activity (26 and 16% of EPEC were intracellular at 120 and 180 min, respectively). This was in contrast to *cfm*, as increased adherence did not result in increased antiphagocytic activity. The percentages of intracellular *cfm* remained similar at 60, 120, and 180 min after bacterial addition.

The above results correlate antiphagocytic activity with in-

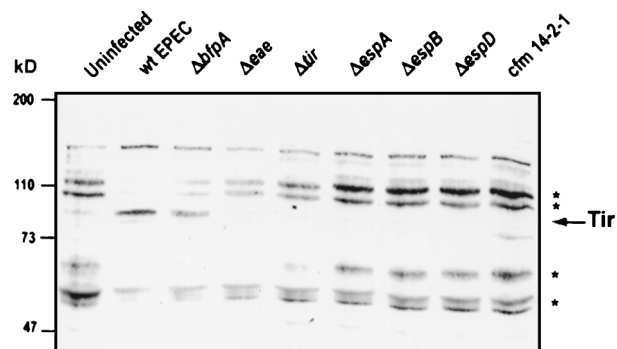


FIG. 3. Signaling-competent EPEC induces tyrosine dephosphorylation in J774 cells. J774 cells were infected with various EPEC strains for 3 h prior to isolation of J774 cell proteins as described in Materials and Methods. Samples were resolved by SDS-8% PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine antibodies. Molecular masses are indicated on the left. *, proteins that are tyrosine dephosphorylated in response to signaling-competent EPEC infection. The arrow indicates the phosphorylated form of Tir. wt, wild type.

creased numbers of adherent secretion-competent EPEC. To further examine this relationship, J774 cells were infected for 180 min with wild-type EPEC and *cfm*, and the percentages of intracellular bacteria were determined for cells with 0 to 5, 5 to 15, or 15 to 50 cell-associated bacteria. The antiphagocytic effect of wild-type EPEC was increased significantly ($P < 0.05$) with increasing numbers of bacteria per J774 cell (Fig. 2C). This differed from *cfm*, as the percentage of intracellular bacteria remained independent of the number of cell-associated bacteria.

Phosphorylation profiles of EPEC-infected J774 cells. To determine whether EPEC induced tyrosine dephosphorylation in J774 cells, similar to that seen with HeLa cells (16), J774 cells were infected with wild-type and mutant EPEC, and protein extracts were resolved by SDS-PAGE. Phosphotyrosine profiles were visualized by Western blot analysis with antiphosphotyrosine antibodies (see Materials and Methods). Wild-type EPEC and the Bfp mutant induced tyrosine dephosphorylation of a triplet of proteins migrating around 50 kDa, a 60-kDa protein, a 100-kDa protein, and a 110-kDa protein (Fig. 3). The *eae* and *tir* mutants induced dephosphorylation of the 60-kDa protein as well as the 50, 100, and 110-kDa proteins, although to a lesser extent. The secretion-deficient mutants (*espA*, *espB*, and *espD* mutants and *cfm*) did not cause tyrosine dephosphorylation of host proteins relative to the uninfected control. The strains that caused dephosphorylation were the same as those that blocked bacterial uptake, correlating dephosphorylation with the observed antiphagocytic phenotype. Interestingly, the *eae* and *tir* mutants, which demonstrated intermediate antiphagocytic effects, induced intermediate levels of dephosphorylation.

Tyrosine dephosphorylation kinetics of EPEC-infected J774 cells. To correlate the onset of antiphagocytosis with tyrosine dephosphorylation, tyrosine phosphorylation profiles were determined for J774 cells infected with wild-type EPEC and *cfm* for 60, 120, and 180 min (Fig. 4). At 60 min of infection, both EPEC- and *cfm*-infected cells showed tyrosine phosphorylation profiles similar to the uninfected control. However, at 120 and 180 min of infection, significant tyrosine dephosphorylation of the 110, 100, 60, and 50-kDa proteins had occurred in the EPEC-infected samples relative to the uninfected control. No such dephosphorylation was observed with the *cfm*-infected cells at 180 min. Tir phosphorylation was observed at

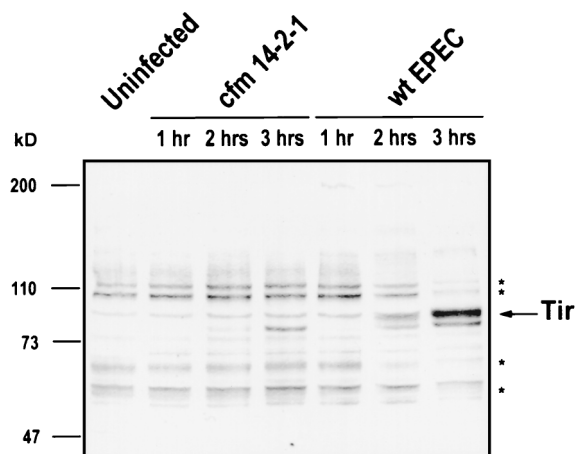


FIG. 4. EPEC-induced tyrosine dephosphorylation is evident after 120 min of infection. Monolayers were left uninfected or were infected with wild-type (wt) EPEC or *cfm* for 60, 120, and 180 min prior to isolation of J774 cell proteins as described in Materials and Methods. Samples were resolved by SDS-8% PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine antibodies. Molecular masses of proteins are indicated on the left. *, proteins that are tyrosine dephosphorylated in response to signaling-competent EPEC infection. The arrow indicates the phosphorylated form of Tir.

120 min and remained at 180 min. These data further suggest a role for tyrosine dephosphorylation in EPEC-mediated antiphagocytosis.

Inhibition of protein tyrosine phosphatases by pervanadate.

To determine if the antiphagocytic phenotype was dependent upon tyrosine dephosphorylation, pervanadate was used to inhibit protein tyrosine phosphatases in J774 cells. Monolayers were infected with EPEC for 90 min prior to the addition of pervanadate for the remaining 60 min of infection, as described in Materials and Methods. No significant loss of membrane integrity was observed during these experiments, as determined by trypan blue staining. EPEC-infected J774 cells treated with pervanadate showed a 3.5-fold increase in the percentage of total cell-associated EPEC organisms that were intracellular (83%) relative to cells that received no treatment (24%) (Fig. 5). However, the *cfm* EPEC-infected cells maintained similar numbers of intracellular bacteria with or without pervanadate treatment (88 and 86%, respectively). The *Y. pseudotuberculosis* wild-type strain was used as a control to demonstrate the ability of pervanadate to inhibit *Y. pseudotuberculosis* antiphagocytic activity (15% of *Y. pseudotuberculosis* organisms were intracellular without pervanadate treatment; 60% of the bacteria were intracellular with pervanadate treatment [data not shown]). Additionally, the tyrosine phosphorylation profile of EPEC-infected cells with pervanadate showed strong inhibition of dephosphorylation compared to the untreated control (data not shown).

Tyrosine phosphatase activity of EPEC-secreted proteins.

To determine if the observed tyrosine dephosphorylation was caused by one of the EPEC-secreted proteins, phosphatase activity was monitored (Table 2). No significant decrease in absorbance was detected when the EPEC-secreted proteins were added to a tyrosine-phosphorylated substrate in vitro compared to the substrate alone, suggesting that tyrosine dephosphorylation did not occur under these conditions. However, very significant phosphatase activity ($P < 0.01$) was detected with the *Y. pseudotuberculosis*-secreted proteins, as would be expected due to the presence of YopH. The addition of pervanadate caused a significant increase in absorbance with *Y. pseudotuberculosis* ($P < 0.01$), suggesting that pervanadate

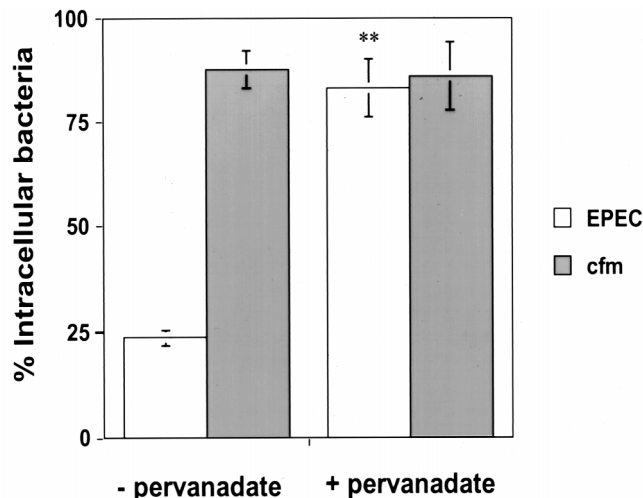


FIG. 5. EPEC-mediated antiphagocytosis is dependent upon tyrosine dephosphorylation. J774 cells were infected with EPEC or *cfm* for 90 min, prior to a 60-min incubation with 0.1 mM VO_4 and 2 mM H_2O_2 . Data are averages and standard errors of the means for three independent experiments performed in duplicate. **, significant difference from the wild type at $P < 0.01$, as obtained by Student *t* tests.

inhibited phosphatase activity of the Yop proteins. No effect was seen with the EPEC-secreted proteins upon pervanadate addition.

DISCUSSION

This study demonstrates that EPEC inhibits its own phagocytosis into cultured macrophage-like cells. This ability is dependent upon the EPEC type III secretion system and its secreted proteins (EspA, EspB, and EspD), which mediate effects on tyrosine-phosphorylated proteins within the host cell. Two lines of evidence suggest the involvement of the secreted proteins. Mutations in the secretion apparatus, as seen in the *cfm* strain, resulted in the inability of EPEC to inhibit phagocytosis by J774 cells. EPEC strains lacking EspA, EspB, or EspD also lost any antiphagocytic capacity, indicating a role in mediating this process, either directly as effector molecules or indirectly as part of a transport delivery apparatus. Time course data indicate that EPEC-mediated antiphagocytosis does not occur within the first hour of infection but is evident after two hours. Protein secretion and significant bacterial attachment do not occur until 90 min after infection (14). Indeed, only low-level adherence to J774 cells was observed until 120 min after bacterial addition, and as bacterial

TABLE 2. EPEC-secreted proteins have no detectable protein tyrosine phosphatase activity in vitro

Sample	Absorbance (405 nm) ^a
None	1.190 ± 0.063
Yop proteins from <i>Y. pseudotuberculosis</i>	0.334 ± 0.023*
Yop proteins from <i>Y. pseudotuberculosis</i> + pervanadate	0.715 ± 0.164*
EPEC-secreted proteins	1.223 ± 0.032
EPEC-secreted proteins + pervanadate	1.258 ± 0.010

^a Values are means ± standard errors of the means (three samples per group). *, significantly different ($P < 0.01$) from value for control group with no sample, as calculated by Student *t* tests.

adherence increased, antiphagocytosis also increased, correlating EPEC attachment and protein secretion/transfer with antiphagocytic activity.

The intermediate antiphagocytic phenotype observed for the Tir and intimin mutants correlated with intermediate dephosphorylation of the same host proteins that are dephosphorylated following infection with both wild-type and Bfp mutant strains. Tir and intimin mutants thus seem to be less efficient at inducing antiphagocytosis although they are still signal-competent, unlike the EspA, EspB, EspD, or cfm mutants. This argues for a role of intimate attachment in antiphagocytosis. Prolonged intimate attachment of EPEC to the host cell leads to cytoskeletal rearrangements and pedestal formation. The role of tyrosine dephosphorylation in pedestal formation remains unclear. Prolonged treatment of EPEC-infected J774 cells with pervanadate leads to cell death (data not shown), and as a consequence it cannot be determined if pedestals are formed in the presence of this phosphatase inhibitor. Therefore, it cannot be ruled out that cytoskeletal rearrangements are not involved in the EPEC-mediated antiphagocytic process. Another explanation would be that intimate attachment enhances translocation of bacterial effectors required for inducing antiphagocytosis, since it has been shown that EspB is less efficiently translocated into HeLa cells by the intimin mutant than by the wild-type strain (31).

The number of cell-associated bacteria also affected the levels of antiphagocytic activity, probably through a dose effect on the signaling events triggered by the secreted proteins. It might be expected, therefore, that microcolony formation, mediated by Bfp, would be important in the process by increasing the number of adherent bacteria and thereby delivering a stronger antiphagocytic signal. However, there were no differences in levels of uptake between the wild type and the *bfp* mutant, suggesting that this was not true. Microcolony formation could possibly function in antiphagocytosis by interconnecting large numbers of bacteria to the macrophage surface. However, the secretion mutants are still capable of microcolony formation, and very few bacteria are found extracellularly on J774 cells, suggesting that this is probably not the case.

Antiphagocytic activity was correlated with tyrosine dephosphorylation events, and the presence of pervanadate specifically inhibited the antiphagocytic activity of wild-type EPEC without hindering the uptake of the signaling mutant, cfm. Pervanadate also inhibited the antiphagocytic activity of *Y. pseudotuberculosis*, as previously reported (2). Since *Y. pseudotuberculosis* transfers a phosphatase into host cells that leads to the activity, we hypothesized that EPEC may also inject a phosphatase. However, no tyrosine phosphatase activity was detected with the secreted proteins with an in vitro assay system. This suggests that EPEC, unlike *Y. pseudotuberculosis*, does not secrete a functional phosphatase under these conditions. It is possible that EPEC may secrete a phosphatase that requires a eukaryotic cofactor for activity. Alternatively, the secreted proteins may trigger the activation of a mammalian tyrosine phosphatase as part of their signal activation.

Taken together, the present findings demonstrate that EPEC blocks its own uptake into phagocytic cells by a mechanism associated with tyrosine dephosphorylation of host proteins. The EPEC-induced dephosphorylation events are dependent upon the secretion of several bacterial proteins, whose specific functions remain undefined. Research is ongoing to further elucidate the mechanism by which EPEC mediates its antiphagocytic phenotype.

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REFERENCES

1. Abe, A., B. Kenny, M. Stein, and B. B. Finlay. 1997. Characterization of two virulence proteins secreted by rabbit enteropathogenic *Escherichia coli*, EspA and EspB, whose maximal expression is sensitive to host body temperature. *Infect. Immun.* **65**:3547–3555.
2. Andersson, K., N. Carballeira, K. E. Magnusson, C. Persson, O. Stendahl, H. Wolf-Watz, and M. Fallman. 1996. YopH of *Yersinia pseudotuberculosis* interrupts early phosphotyrosine signaling associated with phagocytosis. *Mol. Microbiol.* **20**:1057–1069.
3. Baldwin, T. J., W. Ward, A. Aitken, S. Knutton, and P. H. Williams. 1991. Elevation of intracellular free calcium levels in HEp-2 cells infected with enteropathogenic *Escherichia coli*. *Infect. Immun.* **59**:1599–1604.
4. Bliska, J. B., K. L. Guan, J. E. Dixon, and S. Falkow. 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. *Proc. Natl. Acad. Sci. USA* **88**:1187–1191.
5. Bölin, I., L. Norlander, and H. Wolf-Watz. 1982. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. *Infect. Immun.* **37**:506–512.
6. Donnenberg, M. S., S. B. Calderwood, A. Donohue-Rolfe, G. T. Keusch, and J. B. Kaper. 1990. Construction and analysis of *TnphoA* mutants of enteropathogenic *Escherichia coli* unable to invade HEp-2 cells. *Infect. Immun.* **58**:1565–1571.
7. Donnenberg, M. S., and J. B. Kaper. 1991. Construction of an *eaeA* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect. Immun.* **59**:4310–4317.
8. Dytoc, M., L. Fedorko, and P. M. Sherman. 1994. Signal transduction in human epithelial cells infected with attaching and effacing *Escherichia coli* in vitro. *Gastroenterology* **106**:1150–1161.
9. Foubister, V., I. Rosenshine, and B. B. Finlay. 1994. A diarrheal pathogen, enteropathogenic *Escherichia coli* (EPEC), triggers a flux of inositol phosphates in infected epithelial cells. *J. Exp. Med.* **179**:993–998.
10. Foubister, V., I. Rosenshine, M. S. Donnenberg, and B. B. Finlay. 1994. The *eaeB* gene of enteropathogenic *Escherichia coli* is necessary for signal transduction in epithelial cells. *Infect. Immun.* **62**:3038–3040.
11. Gomez-Duarte, O. G., and J. B. Kaper. 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. *Infect. Immun.* **63**:1767–1776.
12. Inman, L. R., and J. R. Canteley. 1983. Specific adherence of *Escherichia coli* (strain RDEC-1) to membranous (M) cells of the Peyer's patch in *Escherichia coli* diarrhea in the rabbit. *J. Clin. Invest.* **71**:1–8.
13. Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper. 1990. A genetic locus of enteropathogenic *E. coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc. Natl. Acad. Sci. USA* **87**:7839–7843.
14. Kenny, B., and B. B. Finlay. 1995. Protein secretion by enteropathogenic *Escherichia coli* is essential for transduction signals to epithelial cells. *Proc. Natl. Acad. Sci. USA* **92**:7991–7995.
15. Kenny, B., L. C. Lai, B. B. Finlay, and M. S. Donnenberg. 1996. EspA, a protein secreted by enteropathogenic *Escherichia coli*, is required to induce signals in epithelial cells. *Mol. Microbiol.* **20**:313–323.
16. Kenny, B., and B. B. Finlay. 1997. Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C- γ 1. *Infect. Immun.* **65**:2528–2536.
17. Kenny, B., A. Abe, M. Stein, and B. B. Finlay. 1997. Enteropathogenic *Escherichia coli* protein secretion is induced in response to conditions similar to those found in the gastrointestinal tract. *Infect. Immun.* **65**:2606–2612.
18. Kenny, B., R. DeVinney, M. Stein, D. J. Reinscheid, E. A. Frey, and B. B. Finlay. 1997. Enteropathogenic *Escherichia coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* **91**:511–520.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
20. Lai, L. C., L. A. Wainwright, K. D. Stone, and M. S. Donnenberg. 1997. A third secreted protein that is encoded by the enteropathogenic *Escherichia coli* pathogenicity island is required for transduction of signals and for attaching and effacing activities in host cells. *Infect. Immun.* **65**:2211–2217.
21. Levine, M. M., and R. Edelman. 1984. Enteropathogenic *Escherichia coli* of classic serotypes associated with infant diarrhea: epidemiology and patho-

- genesis. *Epidemiol. Rev.* **162**:1285–1292.
22. **Ralph, P., and I. Nakoinz.** 1975. Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. *Nature* **257**:393–394.
 23. **Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz.** 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* **15**:261–267.
 24. **Rosenshine, I., M. S. Sonnenberg, J. B. Kaper, and B. B. Finlay.** 1992. Signal exchange between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell protein to initiate cytoskeletal rearrangement and bacterial uptake. *EMBO J.* **11**:3551–3560.
 25. **Rosenshine, I., S. Ruschkowski, M. Stein, D. J. Reinscheid, S. D. Mills, and B. B. Finlay.** 1996. A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J.* **15**:2613–2624.
 26. **Rosqvist, R., I. Bolin, and H. Wolf-Watz.** 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. *Infect. Immun.* **56**:2139–2143.
 27. **Rosqvist, R., A. Forsberg, M. Rimpilainen, T. Bergman, and H. Wolf-Watz.** 1990. The cytotoxic protein YopE of *Yersinia* obstructs the primary defence. *Mol. Microbiol.* **4**:657–667.
 28. **Rosqvist, R., A. Forsberg, and H. Wolf-Watz.** 1991. Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. *Infect. Immun.* **59**:4562–4569.
 29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 30. **Siebers, A., and B. B. Finlay.** 1996. M cells and the pathogenesis of mucosal and systemic infections. *Trends Microbiol.* **4**:22–29.
 31. **Wolff, C., I. Nisan, E. Hanski, G. Frankel, and I. Rosenshine.** 1998. Protein translocation into host epithelial cells by infecting enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **28**:143–155.

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