

Role of Yops and Adhesins in Resistance of *Yersinia enterocolitica* to Phagocytosis

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Yersinia enterocolitica is a pathogen endowed with two adhesins, Inv and YadA, and with the Ysc type III secretion system, which allows extracellular adherent bacteria to inject Yop effectors into the cytosol of animal target cells. We tested the influence of all of these virulence determinants on opsonic and nonopsonic phagocytosis by PU5-1.8 and J774 mouse macrophages, as well as by human polymorphonuclear leukocytes (PMNs). The adhesins contributed to phagocytosis in the absence of opsonins but not in the presence of opsonins. In agreement with previous results, YadA counteracted opsonization. In every instance, the Ysc-Yop system conferred a significant level of resistance to phagocytosis. Nonopsonized single-mutant bacteria lacking either YopE, -H, -T, or -O were phagocytosed significantly more by J774 cells and by PMNs. Opsonized bacteria were phagocytosed more than nonopsonized bacteria, and mutant bacteria lacking either YopH, -T, or -O were phagocytosed significantly more by J774 cells and by PMNs than were wild-type (WT) bacteria. Opsonized mutants lacking only YopE were phagocytosed significantly more than were WT bacteria by PMNs but not by J774 cells. Thus, YopH, -T, and -O were involved in all of the phagocytic processes studied here but YopE did not play a clear role in guarding against opsonic phagocytosis by J774. Mutants lacking YopP and YopM were, in every instance, as resistant as WT bacteria. Overexpression of YopE, -H, -T, or -O alone did not confer resistance to phagocytosis, although it affected the cytoskeleton. These results show that YopH, YopT, YopO, and, in some instances, YopE act synergistically to increase the resistance of *Y. enterocolitica* to phagocytosis by macrophages and PMNs.

The genus *Yersinia* includes three species that are pathogenic for rodents and humans, *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, the latter being the most prevalent in humans. These three bacterial species exhibit different degrees of virulence but have in common an ability to multiply in lymphatic tissues and to defeat the primary immune host defenses. This capacity is provided by type III secretion-translocation machinery encoded by a 70-kb plasmid called pYV in *Y. enterocolitica*. This system allows extracellular bacteria adhering to the surfaces of eukaryotic target cells to inject bacterial proteins into the cytosol of these cells, thereby disarming them and disrupting their communication network. The injected bacterial effectors are called Yops (YopE, -H, -T, -O, -P, and -M). They are secreted by the Ysc secretion apparatus and cross the eukaryotic cell membrane by a pore formed by the other Yops YopB, YopD, and LcrV (for a review, see reference 20).

Adhesion to eukaryotic target cells is crucial for injection of Yops (14), and in *Y. enterocolitica*, it is mediated by the adhesin YadA (29, 36) or by invasin (Inv) (32). YadA, which is encoded on the pYV plasmid together with the Yops and the Ysc

secretion apparatus, is expressed at 37°C (36). It is primarily an adhesin, but it also has the capacity to promote invasion of epithelial cells (11). In addition to its role as an adhesin, it protects *Y. enterocolitica* against the bactericidal and opsonizing action of complement by binding factor H (17, 18, 51). By this effect, YadA confers some resistance to phagocytosis by human neutrophils (polymorphonuclear leukocytes [PMNs]) and macrophages (17, 59, 66). Inv, which is chromosomally encoded and produced at low temperatures (48), mediates adhesion but also triggers integrin-mediated uptake into nonphagocytic mammalian cells (for a review, see reference 33), promotes self-association (22), and triggers interleukin-8 release (62). In the absence of opsonization, Inv triggers internalization in macrophages via $\beta 1$ integrins, Cdc42Hs, and WASp (41, 68). The Yop effectors modulate eukaryotic signal transduction pathways. Four of them modify the actin cytoskeleton dynamics. YopH is a tyrosine phosphatase that dephosphorylates focal adhesion kinase (Fak), paxillin, Fyn-binding protein (FYB), p130^{cas}, and SKAP-HOM (4, 7, 9, 15, 28, 49, 50). These eukaryotic proteins regulate the association of actin filaments to the cytoplasmic domain of integrin receptors in focal adhesion complexes (15). YopE causes depolymerization of actin stress fibers by activating the Rho GTPase (8, 57, 67). In addition, it has been proposed to specifically modulate Rac-dependent structures in endothelial cells (5). YopT disrupts the actin stress fibers of the cytoskeleton (31) by an undefined modification of the small GTP-binding protein RhoA and its subsequent redistribution from the membrane to the cytosol

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype	Reference
Strains		
E40	<i>Y. enterocolitica</i> O:9	
MRS40	<i>blaA luxAB</i> ⁺ mutant of E40	60
APB40	<i>inv::pMS154</i> mutant of MRS40	14
pYV40 plasmids		
pYV40	WT plasmid of <i>Y. enterocolitica</i> E40	60
pAB404	<i>yopE</i> ₂₁ <i>yopH</i> _{Δ1-352}	13
pAB406	<i>yopO</i> _{Δ65-558}	44
pAB408	<i>yopM</i> ₂₃	44
pAB4052	<i>yopE</i> ₂₁	44
pAPB4011	<i>yadA</i> _{Δ68-326}	14
pIM409	<i>yopT</i> ₁₃₅	31
pIM424	<i>yopE</i> ₂₁ <i>yopT</i> ₁₃₅	31
pIM425	<i>yopH</i> _{Δ1-352} <i>yopT</i> ₁₃₅	14
pIM426	<i>yopE</i> ₂₁ <i>yopH</i> _{Δ1-352} <i>yopT</i> ₁₃₅	14
pIML421	<i>yopE</i> ₂₁ <i>yopH</i> _{Δ1-352} <i>yopP</i> ₂₃ <i>yopM</i> ₂₃ <i>yopT</i> ₁₃₅ <i>yopO</i> _{Δ65-558} (called ΔHOPEMT)	31
pMSK41	<i>yopP</i> ₂₃	44
pMSL41	<i>yscN</i> _{Δ169-177}	63
pPW401	<i>yopB</i> _{Δ89-217}	13
pSI4008	<i>yopH</i> _{Δ1-352}	44
Expression plasmids		
pIML279	pBC18R <i>Plac yopT</i> ⁺ <i>sycT</i> ⁺	31
pMSL34	pSelect-1 <i>PyopO yopO</i> ⁺	This work
pMSL68	pBC19R <i>Plac yopE</i> ⁺ <i>sycE</i> ⁺	This work
pMSL69	pBluescriptKS(-) <i>Plac yopH</i> ⁺ <i>sycH</i> ⁺	This work
Suicide plasmids		
pINT192	<i>oriR6K mobRP4 cat</i> ; contains 1.4-kb <i>NdeI-BglII</i> internal fragment of <i>inv</i> from <i>Y. enterocolitica</i> 8081	48
pMS154	pKNG160 containing 1.4-kb internal <i>inv</i> fragment subcloned from pINT192	64

(31, 69). YopO, called YpkA in *Y. pseudotuberculosis* and *Y. pestis*, is an autophosphorylating serine/threonine kinase that is activated by contact with filamentous actin and phosphorylates it (23, 34). YpkA/YopO also physically interacts with RhoA and Rac, like a RhoA/Rac-binding kinase, resulting in decreased levels of activated RhoA (23). When YpkA/YopO is injected into macrophages, the cells round up but do not detach from the extracellular matrix (27).

Monocytes-macrophages and PMNs represent the first defense mechanisms used by an infected organism against invading bacteria. Two major distinct pathways are involved in the internalization of serum-opsonized particles (1, 2). Both of them are sustained by the Rho GTPase system involved in the reorganization of the actin cytoskeleton (for a review, see reference 16). The first pathway recognizes immunoglobulin G (IgG)-coated particles, on the basis of the immunoglobulin receptor FcγR, and involves actin reorganization mediated by CDC42 and Rac GTPases. The second pathway concerns C3b-opsonized particles, recognized by complement receptor 3, and involves a remodeling of the cytoskeleton sustained by the Rho GTPases (26). Nonopsonic phagocytosis of microorganisms is also very active in phagocytes. Most of the time, it involves lectin-sugar recognition, which also leads to remodeling of F actin mediated by Rho GTPases (6, 37). Early reports on *Y. enterocolitica* pathogenesis showed that the pYV plasmid confers the capacity to resist phagocytosis by macrophages or PMNs in vitro (39, 40, 52) YopE and YopH were later shown

to be two effectors protecting *Y. pseudotuberculosis* and *Y. enterocolitica* from phagocytosis by cultured macrophages (3, 25, 28, 50, 56, 57) or PMNs (4, 65). The two other Yops affecting cytoskeleton dynamics, YopO and YopT, have never been shown to have an antiphagocytic role. In addition, few studies have investigated the role of the adhesins Inv and YadA in phagocytosis by PMNs (17, 54, 66). With some exceptions (25, 59, 65), most of the experiments on phagocytosis were carried out without opsonization. In the present study, we investigated whether YopT and YpkA/YopO are also involved in resistance to phagocytosis. We thus quantified the phagocytosis of an array of Yop effector knockout mutants. We also studied the role of Inv and YadA in phagocytosis. This study was carried out in the absence and in the presence of IgG, complement, or both with freshly isolated human PMNs and with two macrophage cell lines, PU5-1.8 and J774.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Y. enterocolitica* MRS40 is a *blaA* mutant of strain E40 in which the gene encoding β-lactamase A was replaced with the *luxAB* genes (35, 60). The bacterial strains and plasmids used in this study are listed in Table 1. The strains were routinely grown in tryptic soy broth (Oxoid) and plated on tryptic soy agar (Oxoid) containing the required antibiotics. Selective agents were used at the following concentrations: ampicillin, 200 μg/ml; tetracycline, 10 μg/ml; nalidixic acid, 35 μg/ml; kanamycin, 50 μg/ml; Na⁺ arsenite, 1 mM (46). For in vitro induction of the Yop virulon and secretion, *Y. enterocolitica* was grown in brain heart infusion broth (BHI; Remel, Lenexa, Kans.) supplemented with 4 mg of glucose per ml, 20 mM MgCl₂, and

20 mM sodium oxalate (BHI-Ox). Cultures were incubated for 2 h at room temperature and then shifted to 37°C for 4 h (19, 43).

Molecular biology techniques. Yops were precipitated from culture supernatants with 10% (wt/vol) trichloroacetic acid, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and, when appropriate, transferred to nitrocellulose membranes. Immunoblots were developed by using a secondary antibody conjugated to horseradish peroxidase (1:2,000; Dako) and the chemiluminescent substrate Supersignal (Pierce, Rockford, Ill.).

Eukaryotic cell line culture and infection conditions. PU5-1.8 and J774 A1 mouse monocyte macrophages were grown routinely in RPMI 1640 medium, while Rat-1 fibroblasts were grown in Dulbecco modified Eagle medium supplemented with Na-pyruvate at 1 mM (Life Technologies, Merelbeke, Belgium), all at 37°C in a humidified atmosphere under 5% CO₂. Both media were supplemented with 2 mM L-glutamine (Seromed), 10% (vol/vol) fetal bovine serum (FBS), 100 U of penicillin per ml, and 100 µg of streptomycin (Life Technologies) per ml. Twenty hours before infection, PU5-1.8 macrophages, J774 macrophages, and Rat-1 fibroblasts were seeded (10⁷ cells/dish) into 1-cm-diameter tissue culture plates (Greiner, Wemmel, Belgium) unless stated otherwise. Thirty minutes before infection, the cells were washed twice and covered with RPMI 1640 medium or Dulbecco modified Eagle medium lacking FBS and antibiotic. Before infection, overnight cultures of *Y. enterocolitica* were inoculated in BHI at an optical density at 600 nm of 0.2 and grown for 2 h at 25°C and for 30 min at 37°C with shaking. After washing in prewarmed phosphate-buffered saline (PBS), bacteria were added to the cells at a multiplicity of infection (MOI) of 50:1.

Preparation of human neutrophils and infection conditions. Neutrophils were isolated from the blood of healthy donors, separated by the dextran-Ficoll method (Amersham Pharmacia, Uppsala, Sweden) as described in reference 45, resuspended in minimal essential medium–10 mM HEPES (pH 7.4), and maintained for 20 min at 37°C prior to infection. Before infection, overnight cultures of *Y. enterocolitica* were inoculated in BHI at an optical density at 600 nm of 0.2 and grown for 2 h at 25°C and for 30 min at 37°C with shaking. After washing in prewarmed PBS, bacteria were added to the cells at an MOI of 50:1.

Yop translocation experiments. Twenty hours before infection, J774 macrophages were seeded (10⁷ cells/dish) into 10-cm-diameter tissue culture plates (Greiner). Thirty minutes before infection, the cells were washed and covered with RPMI 1640 medium lacking FBS and antibiotic but containing cytochalasin D (5 µg/ml; stock solution, 2 mg/ml in dimethyl sulfoxide; Sigma Aldrich, Bornem, Belgium). Bacteria preincubated as described before were then added at an MOI of 50:1. After an infection period of 2 h at 37°C under 5% CO₂, the culture supernatant was carefully removed and 1 ml of ice-cold extraction buffer (0.1% [vol/vol] Triton X-100 in PBS, 1 mM phenylmethylsulfonyl fluoride, 1 µg of leupeptin per ml, 1 mg of pepstatin per ml) prepared freshly before extraction (Sigma Aldrich) was added to lyse the cells. The cell lysate was centrifuged at 158 × g for 15 min to collect the Triton-insoluble fraction. The supernatant was transferred to a new tube, and the centrifugation was repeated. The supernatant proteins were precipitated by the addition of trichloroacetic acid (Merck Euro-lab, Leuven, Belgium) at a final concentration of 10% (wt/vol). Supernatant and total cells proteins were resuspended in SDS-PAGE loading buffer and analyzed by Western blotting with polyclonal antibodies directed against YopH, -T, and -O and SycE and monoclonal antibody 13A9 directed against YopE.

Oponization with immune serum. Two kinds of sera were used to opsonize bacteria. For phagocytosis by mouse cell lines, bacteria were opsonized with serum from a rabbit immunized by intravenous injections of heat-killed *Y. enterocolitica* O:9 (gift from G. Wauters, Brussels, Belgium). For phagocytosis by human PMNs, bacteria were opsonized with clinical samples of serum that were found to contain anti-*Y. enterocolitica* O:9 antibodies as detected by using the Sanofi–Pasteur–Bio-Rad kit (gift from G. Bigaignon, Brussels, Belgium). For specific IgG opsonization, serum was decanted after 30 min at 56°C. For specific complement opsonization, bacteria were incubated with guinea pig complement (1/500; Sigma Aldrich). To opsonize bacteria, they were grown as explained before, washed in PBS, and incubated for 30 min at 37°C in serum diluted in PBS. Rabbit antiserum was diluted 1/500, while human serum was diluted 1/800. Opsonized bacteria were washed twice with PBS before infection. Opsonization of *Y. enterocolitica* was monitored by staining with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and anti-human C3c reacting with human complement factors C3c and C3bi (Prosan, Merelbeke, Belgium). Strong staining of the serum-opsonized bacteria with FITC-conjugated anti-IgGs revealed the presence of IgG on the bacterial surface. Similarly, strong staining of the serum-opsonized bacteria with FITC-conjugated anti-C3c revealed the presence of C3bi on the bacterial surface. Survival of bacteria after 30 min of incubation with dilute immune serum was checked by plating dilutions of opso-

nized and nonopsonized bacteria on tryptic soy broth. Similar numbers of colonies appeared on both plates after 24 h of incubation at 25°C.

Phagocytosis assay for macrophage cell lines. Bacteria and cells were cultured and placed in contact as described before. After an infection period of 30 min, bacteria associated with the target cells in intra- and extracellular locations were distinguished by the double-immunofluorescence method as described previously (30, 55). To stain extracellularly located bacteria, the coverslips were first washed with PBS and then incubated for 30 min at room temperature with rabbit anti-*Y. enterocolitica* O:9 LPS serum (diluted 1:500). Thereafter, the excess antiserum was removed by four washes in PBS and the coverslips were covered with tetramethyl rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Texas Red; 12 µg/ml; Molecular Probes, Leiden, The Netherlands). After incubation for 20 min at 37°C, cells were washed four times in PBS. Coverslips were air dried, fixed, permeabilized by treatment with ice-cold methanol for 90 s, and dried again. To stain the intracellular and extracellular bacteria, the coverslips were incubated again with rabbit anti-*Y. enterocolitica* O:9 LPS serum (1:500) for 1 h at 37°C. The coverslips were then washed four times in PBS, overlaid with FITC-conjugated goat anti-rabbit immunoglobulins (24 µg/ml; Prosan), and incubated for 20 min at 37°C. Finally, after another washing, the coverslips were mounted in 50% Mowiol (Polysciences) containing 100 mg of diazabicyclooctane (DABCO) per ml and examined by fluorescence microscopy. Under these conditions, the extracellular bacteria were stained with both FITC and Texas Red and appeared green or red, depending on the filter used, while intracellular bacteria were stained only with FITC and appeared green. At least 100 cells were examined, and the number of extracellularly located bacteria and the total number of cell-associated bacteria were determined. For each condition, the experiments were done three times in duplicate. The phagocytosis percentage represents the ratio of the number of intracellular bacteria to the total number of cell-associated bacteria.

Phagocytosis assay for PMNs. For monitoring of phagocytosis by PMNs, the protocol was slightly different. Infection was carried out for 30 min at 37°C at an MOI of 50:1 as previously described (37). The neutrophils were then washed to remove free-floating bacteria and fixed for 30 min at room temperature with 3.7% paraformaldehyde in PBS containing 15 mM sucrose (pH 7.4). After neutralization with 50 mM NH₄Cl, the coverslips were washed with PBS and incubated for 30 min with rabbit anti-*Y. enterocolitica* O:9 LPS serum (diluted 1:500) and then for 30 min with goat anti-rabbit Texas Red antibodies (12 µg/ml) to stain the extracellular bacteria. The cells were then permeabilized by 2 min of treatment at room temperature with 0.5% Triton X-100 in PBS and subsequently washed in PBS. Intracellular and extracellular bacteria were then stained by sequential addition of anti-*Y. enterocolitica* O:9 LPS serum (1:100) and goat anti-rabbit antibodies coupled to FITC (24 µg/ml). The coverslips were air dried, mounted on Mowiol-DABCO, and examined by fluorescence microscopy.

Immunofluorescence staining and confocal microscopy analysis. Rat-1 fibroblasts were grown as monolayers on coverslips and infected with *Y. enterocolitica* at an MOI of 50:1. After 2 h 30 min of infection, the first sign of cytotoxicity appeared and the cells were fixed in 2% paraformaldehyde for 20 min. After washing with PBS, membranes were permeabilized with PBS–0.5% Triton X-100 for 10 min. Actin microfilaments were labeled by incubation for 40 min at 37°C with FITC-conjugated phalloidin. Samples were mounted in (Mowiol-DABCO), and cells were examined on a confocal laser scanning microscope equipped with dual detectors and an argon-krypton laser (MRC 1024; Bio-Rad Laboratories).

Statistical analysis. The number of samples in each experimental condition is indicated in the figure legends. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple-comparison test.

RESULTS

Resistance of *Y. enterocolitica* MRS40 to phagocytosis by J774 cells and PMNs. We first monitored the behavior of *Y. enterocolitica* MRS40 in contact with J774 mouse macrophage-like cells at an MOI of 50:1. After 30 min of infection at 37°C, we counted the cell-associated bacteria and calculated the percentage of bacteria that were phagocytosed by using the double-immunofluorescence method (30). In parallel, we tested wild-type (WT) *Y. enterocolitica* and a mutant deprived of YscN, the ATPase of the type III Ysc machinery. Adherence of both nonopsonized bacteria to J774 was in the same range (Fig. 1A). The WT bacteria adhered somewhat better, presum-

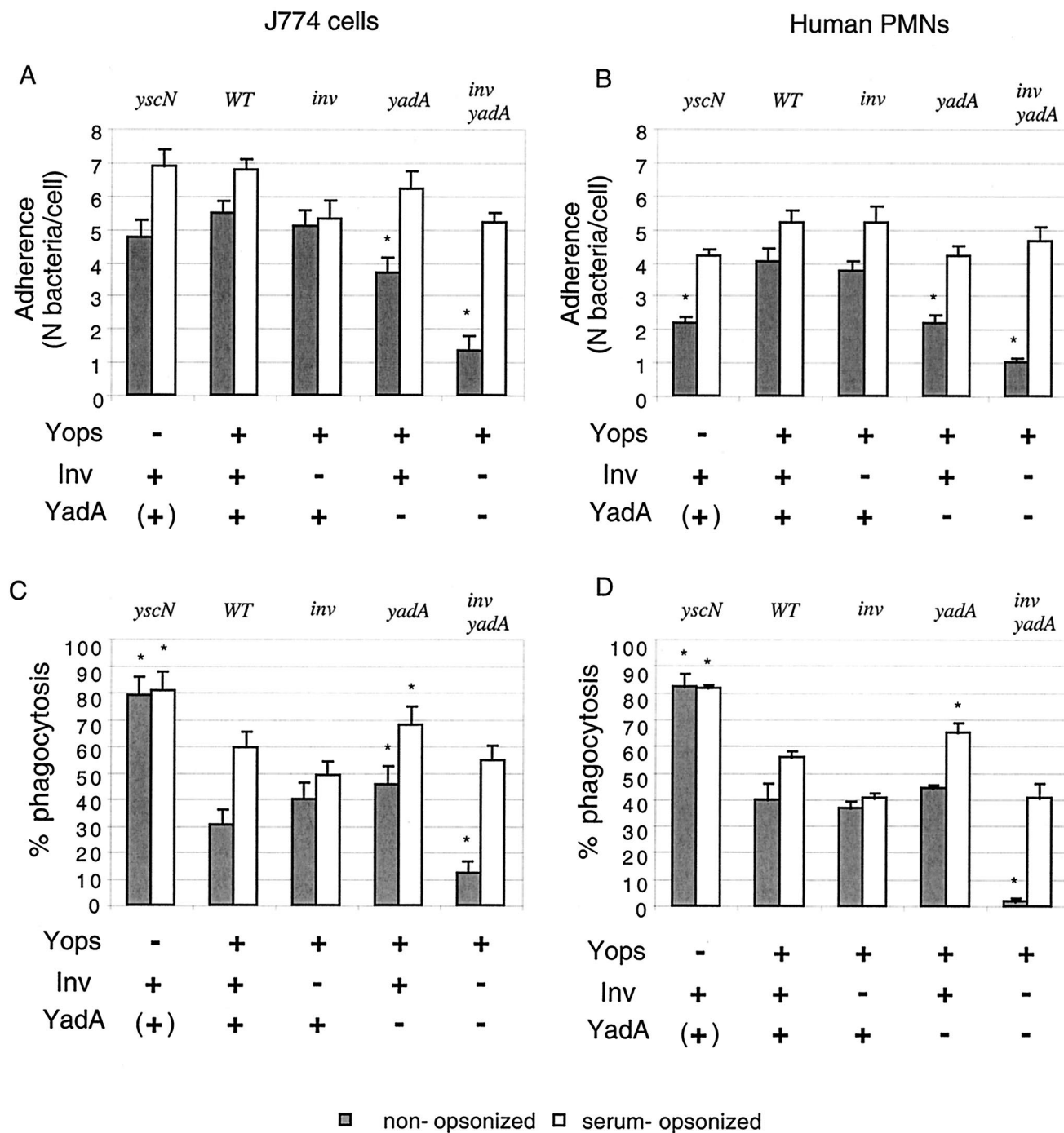


FIG. 1. (A and B) Influence of Inv and YadA on adherence and phagocytosis of *Y. enterocolitica* MRS40 by J774 macrophages (A and C) and human PMNs (B and D). Nonopsonized or serum-opsonized WT *Y. enterocolitica* [MRS40(pYV40)], the *yscN* mutant [MRS40(pMSL41)], the *inv* mutant [APB40(pYV40)], the *yadA* mutant [MRS40(pAPB4011)], and the *inv yadA* mutant [APB40(pAPB4011)] were exposed to J774 cells (A and C) or PMNs (B and D) at a calculated MOI of 50:1 for 30 min at 37°C. The number of adherent bacteria per cell and the percentage of phagocytosis were determined by the double-immunofluorescence technique. Results of adherence are given as the mean plus the standard error of the mean. Phagocytosis results are given as percentages of phagocytosed bacteria relative to the total number of cell-associated bacteria (*, $P < 0.05$ compared to the WT; $n = 6$). The values are the mean plus the standard error of the mean. For each experiment, at least 100 cells were examined.

ably because they produce more YadA (42). However, only 30% of the WT *Y. enterocolitica* MRS40 bacteria associated with J774 cells were ingested (Fig. 1C) while 80% of the cell-associated *yscN* bacteria were taken up. This demonstrated

that the Ysc-Yop system allows *Y. enterocolitica* MRS40 to resist phagocytosis by J774 cells. Since *Y. enterocolitica* encounters not only macrophages but also PMNs during infection, we also tested the resistance of *Y. enterocolitica* bacteria to inges-

tion by PMNs freshly isolated from a healthy human donor. As shown in Fig. 1D, only 40% of the WT bacteria were phagocytosed while 82% of the YscN-deficient bacteria were phagocytosed. PMNs were thus more efficient than J774 cells at ingesting *Y. enterocolitica* but, again, the Ysc-Yop-deficient bacteria were more phagocytosed than WT bacteria. This result is in agreement with a previous study (65) and illustrates the reactivity of freshly isolated neutrophils in comparison with immortalized macrophage cell lines.

Role of bacterial adhesins in the internalization process. *Y. enterocolitica* adheres to many different cell types by means of its two adhesins, Inv and YadA. With most cell types, but not with macrophages, the presence of Inv or YadA is an absolute prerequisite for activation of the Ysc-Yop system (14, 68). In the case of phagocytes, the adhesins could have two antagonistic effects. By promoting contact with the phagocyte, they may trigger injection of antiphagocytic Yops but, on the contrary, they could also favor phagocytosis. We thus tested the role of Inv and/or YadA in both adherence and phagocytosis by J774 macrophages and PMNs. This was done by using *inv* or *yadA* single mutants, as well as an *inv yadA* double mutant.

Under our experimental conditions and in the absence of opsonization (Fig. 1A, grey bars), adherence of the *inv* mutant to J774 was almost equivalent to the adherence of WT bacteria (5.1 ± 0.4 and 5.5 ± 0.3 , respectively) and adherence of *yadA* mutant bacteria was slightly decreased (3.7 ± 0.4 ; Fig. 1A, grey bars). In contrast, the adherence of the *inv yadA* double-mutant bacteria was decreased fourfold compared to the adherence of WT bacteria (1.4 ± 0.4). These values confirmed that both adhesins, especially YadA, play a role in the adherence of nonopsonized *Y. enterocolitica* MRS40 to phagocytic cells. Notice that adherence is significantly lower on PMNs than on J774 cells, but the ratio of mutant to WT bacteria is conserved (Fig. 1B, grey bars). Although the double-mutant bacteria adhered less than the other bacteria, they still interacted with phagocytes. This suggests that another element, different from Inv and YadA, also leads to adherence. It could be either a phagocyte receptor acting in the absence of opsonization or another, unidentified, bacterial adhesin.

The percentage of phagocytosis of nonopsonized *inv* or *yadA* single-mutant bacteria by J774 macrophages (Fig. 1C) or by PMNs (Fig. 1D) was not significantly different from the percentage of phagocytosis of WT bacteria. In contrast, the few *inv yadA* double-mutant bacteria that interacted with phagocytic cells were very poorly internalized. This indicates that, in the absence of opsonization, Inv or YadA favors entry into phagocytes. It also indicates that the unidentified adherence mechanism does not trigger efficient uptake of bacteria.

In vivo, recognition of the invading bacteria by phagocytes involves opsonins such as C3bi and IgG. We thus tested the influence of Inv and YadA on adherence and phagocytosis after incubation of bacteria with nonheated immune serum containing opsonins like IgG and C3bi (Fig. 1, white bars). For experiments with J774 mouse cells, bacteria were opsonized with anti-*Y. enterocolitica* O:9 LPS rabbit serum, while for experiments with human PMNs, they were opsonized with human serum containing antibodies directed against *Y. enterocolitica* O:9. Opsonization increased the number of cell-associated bacteria for every strain tested, including the *inv yadA* double mutant (Fig. 1A and B, white bars). These double-

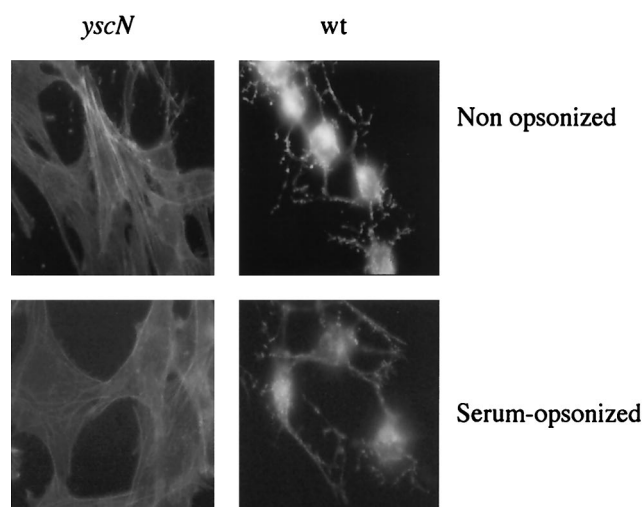


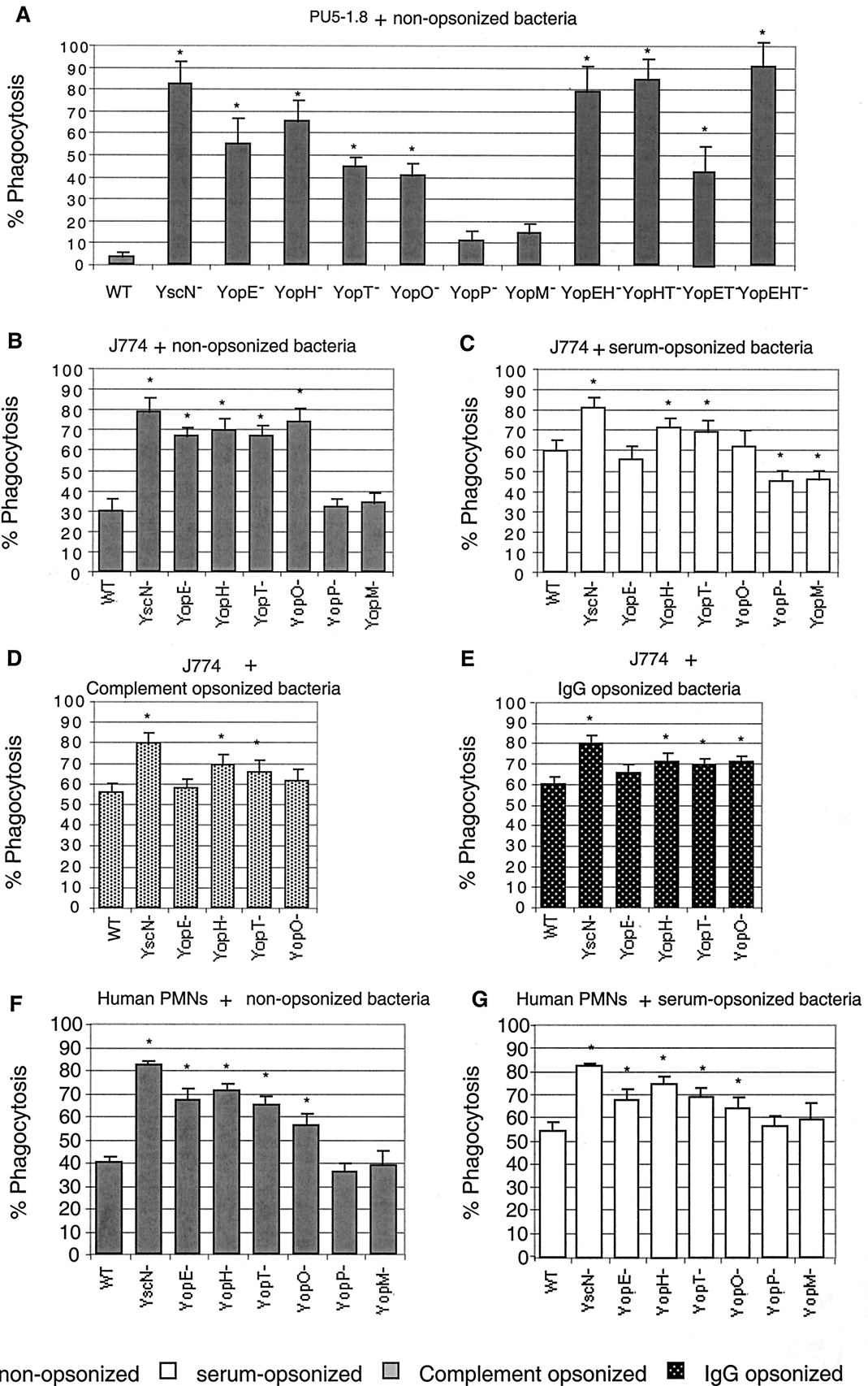
FIG. 2. Cytotoxic effect of serum-opsonized WT *Y. enterocolitica* MRS40 bacteria. Rat-1 fibroblasts were infected with *Y. enterocolitica* [MRS40(pYV40)] bacteria or *yscN* mutant [MRS40(pMSL41)] bacteria, and cytotoxicity was monitored by staining with FITC-phalloidin after 2 h 30 min of infection. Opsonized and nonopsonized WT *Y. enterocolitica* MRS40 bacteria were cytotoxic for Rat-1 fibroblasts, in contrast to opsonized or nonopsonized *yscN* mutant bacteria.

mutant bacteria even associated with phagocytes as efficiently as WT bacteria, which confirmed the efficacy of opsonization by the immune serum. Opsonized *yadA* bacteria were taken-up more efficiently than opsonized *inv* bacteria and opsonized WT bacteria. This presumably results from the capacity of YadA to prevent C3bi deposition on bacteria, as shown by China et al. (18). This protective effect of YadA can also explain the strong increase in the phagocytosis percentage caused by opsonization of *inv yadA* double-mutant bacteria. Indeed, the increase in phagocytosis due to opsonization is much more pronounced for the *inv yadA* double mutant than for the *inv* single mutant.

We concluded from all of this that, in the absence of opsonization, entry of *Y. enterocolitica* into phagocytes is essentially a phenomenon driven by YadA or Inv. In contrast, opsonization leads to phagocytosis independently of YadA and Inv. In the latter situation, YadA tends to reduce phagocytosis. In any instance, *yscN* mutant bacteria were internalized more than WT bacteria, indicating that the Ysc-Yop system counteracts the engulfment mechanism.

Influence of opsonization on Yop injection. It is not known whether opsonization reduces the efficiency of the type III secretion-translocation system. To address this question, we used Rat-1 fibroblasts because they have a developed actin cytoskeleton and because the destruction of the cytoskeleton is the most sensitive test with which to monitor the delivery of Yops. We thus infected Rat-1 cells at an MOI of 50:1, and after 2.5 h of infection, we fixed the cells and stained actin with FITC-phalloidin. As shown in Fig. 2, incubation with rabbit anti-*Y. enterocolitica* O:9 immune serum, used at the same dilution as for opsonization, did not prevent or even delay the destruction of the actin cytoskeleton. Thus, we concluded that serum opsonization does not inhibit the activity of the type III Ysc-Yop system.

Role of Yop effectors in resistance to nonopsonic phagocy-



toxis. To determine the involvement of each Yop effector in resistance to phagocytosis, a collection of six single mutants (Table 1) was used. For each experiment, we measured the total number of bacteria associated with PU5-1.8 macrophages and observed that it was similar for the different strains tested (data not shown). In the absence of opsonization, the *yopE*, *yopH*, *yopT*, and *yopO* single-mutant bacteria were phagocytosed by PU5-1.8 macrophages at least 10-fold more than WT bacteria (Fig. 3A). In contrast, *yopP* and *yopM* single-mutant bacteria resisted phagocytosis as well as did WT bacteria, showing that these effectors do not play any role in resistance to phagocytosis. Double or triple mutants affected in YopE, YopH, and YopT (Fig. 3A) were more phagocytosed than single mutants, as already described for *Y. pseudotuberculosis* (25). The *yopET* double-mutant bacteria seemed to be less phagocytosed than the *yopE* single-mutant bacteria. However, this difference was not significant according to the statistical analysis ($P < 0.05$). We concluded from this that YopE, -H, -T, and -O are all necessary for *Y. enterocolitica* MRS40 to fully resist engulfment by PU5-1.8 macrophages.

yopE, *yopH*, *yopT*, and *yopO* single mutants were also phagocytosed more than WT bacteria by J774 macrophages (Fig. 3B, grey bars). The percentage of phagocytosis increased from 30% (WT) to 66% (*yopE* or *yopT*), 70% (*yopH*), and even 74% (*yopO*), confirming that each of these four effectors contributes significantly to resistance to phagocytosis. In J774 infection, as well as in PU5-1.8 infection, removal of YopP or YopM did not increase the percentage of phagocytosis of cell-associated bacteria compared to WT *Y. enterocolitica* MRS40. Single mutations affecting YopE, YopH, YopT, and YopO also significantly increased phagocytosis by PMNs. Again, mutations affecting YopP and YopM had no effect (Fig. 3F, grey bars). Thus, single mutations in any of the Yop effectors known to act on the cytoskeleton significantly increased the phagocytosis of nonopsonized bacteria, indicating that each of them plays an important role. Single-mutant bacteria were phagocytosed almost as efficiently as bacteria deprived of the whole Yop virulon.

Role of Yop effectors in resistance to opsonic phagocytosis. Phagocytosis of *Y. enterocolitica* by J774 and by PMNs was also monitored after opsonization by immune serum providing both complement and specific IgG. The results obtained with both types of cells were very similar (Fig. 3C and G, white bars). The opsonization of WT *Y. enterocolitica* increased by about 30% the number of bacteria engulfed by J774 and by about 15% the number of bacteria engulfed by PMNs, indicating that WT *Y. enterocolitica* bacteria resist phagocytosis less when they are opsonized. The *yscN* mutant bacteria were still phagocytosed significantly more than WT bacteria, indicating that the Ysc-

Yop virulon also counteracts opsonic phagocytosis. However, the effect of single mutations was not so clear in the presence of opsonins as in the absence of opsonins. Compared to opsonized WT bacteria, opsonized *yopE* and *yopO* mutant bacteria were not phagocytosed significantly more. In contrast, opsonized *yopH* and *yopT* bacteria were phagocytosed slightly but significantly ($P < 0.05$) more than WT bacteria. Since the four Yops involved in phagocytosis resistance control different host cell GTPases that are differentially implicated in IgG or complement opsonic phagocytosis (1, 2), we wondered whether the different Yops could be differently involved in the two types of opsonic phagocytosis. To address this question, we performed phagocytosis experiments with either complement- or IgG-opsonized bacteria. The results, presented in Fig. 3D and E, respectively, were not different from those obtained with double opsonization (Fig. 3C). In both cases, phagocytosis of the *yopH*, *YopT*, and *yopO* mutants was significantly increased compared to that of WT bacteria. Again, no significant difference was observed for the *yopE* mutant.

YopE, -H, -T, or -O alone is not sufficient for resistance to phagocytosis. We have shown that each of the four effectors YopE, -H, -T, or -O contributes to resistance to phagocytosis. To investigate whether each of them could confer some level of protection when overproduced alone, we used a strain mutated in all six effector genes and called Δ HOPEMT (31). We introduced separately into this multimutant strain high-copy plasmids containing *yopE* and its *yscE* chaperone gene, *yopH* and *yscH*, *yopT* and *yscT*, and *yopO* alone. No chaperone could be identified for this Yop (Table 1). We first controlled expression of these different constructions for their capacity to secrete the relevant Yop under calcium chelation conditions. YopE, -H, -T, and -O were clearly visible in the supernatants (Fig. 4C), but, as already observed by Iriarte and Cornelis (31), YopT was detected in the supernatant of the MRS40 Δ HOPEMT-overproducing YopT strains but not in the supernatant of the WT strain. Translocation of these effectors into J774 macrophages was also checked by Triton fractionation of infected cells (Fig. 4D). As seen in Fig. 4D, all four Yops, including YopT, were detected in the Triton-soluble fraction, indicating that all four Yops were indeed injected. After infection with *yopB* mutant bacteria, we could not see any of the four effectors in the Triton-soluble fraction, confirming that we only detected injected proteins. We then compared the phagocytosis of these bacteria producing only one effector to the phagocytosis of Δ HOPEMT. As shown in Fig. 4A and B, after 30 min of contact, there was no significant reduction of phagocytosis by J774 or PMNs due to the injection of only one effector. Thus, we concluded that none of the antiphagocytic Yops alone can confer a significant degree of resistance.

FIG. 3. Phagocytosis of nonopsonized (grey bars, A, B, and F), serum-opsonized (white bars, C and G), complement-opsonized (D), and IgG-opsonized (E) *Y. enterocolitica* MRS40 and effector mutants by PU5-1.8 macrophages (A), J774 macrophages (B, C, D, and E), and PMNs (F and G). *Y. enterocolitica* MRS40(pYV40), the *yscN* mutant [MRS40(pMSL41)], the *yopE* mutant [MRS40(pAB4052)], the *yopH* mutant [MRS40(pSI4008)], the *yopT* mutant [MRS40(pIM409)], the *yopO* mutant [MRS40(pAB406)], the *yopP* mutant [MRS40(pMSK41)], the *yopM* mutant [MRS40(pAB408)], the *yopEH* mutant [MRS40(pAB404)], the *yopHT* mutant [MRS40(pIM425)], the *yopET* mutant [MRS40 (pIM424)], and the *yopEHT* mutant [MRS40(pIM426)] were exposed to phagocytic cells at a calculated MOI of 50:1 for 30 min at 37°C. Phagocytosis percentages were determined by double-immunofluorescence assay. Phagocytosis results are given as percentages of phagocytosed bacteria relative to the total number of cell-associated bacteria (*, $P < 0.05$ compared to the WT; $n = 6$). The values are the mean plus the standard error of the mean. For each experiment, at least 100 cells were counted.

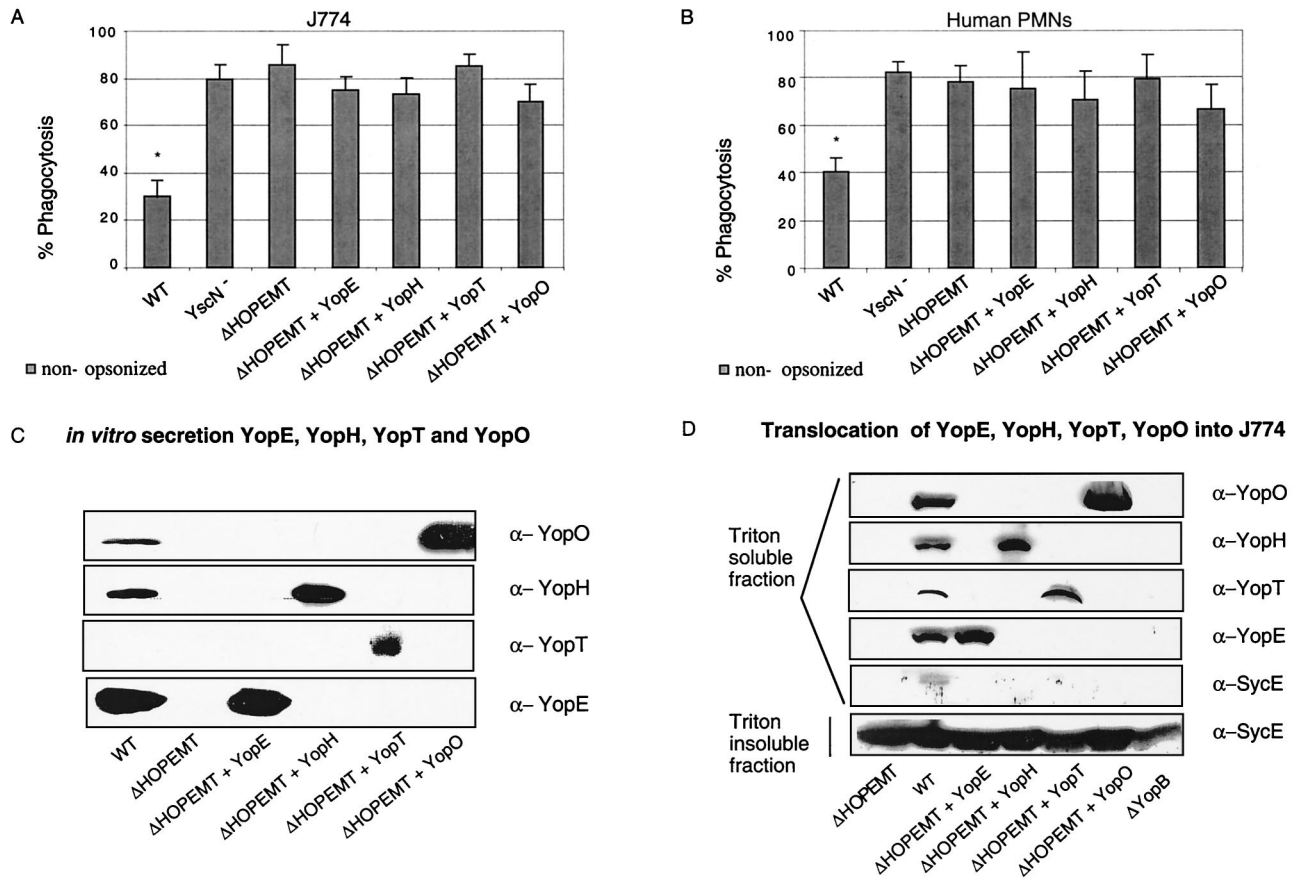


FIG. 4. (A and B) Phagocytosis of the *Y. enterocolitica* ΔHOPEMT mutant overexpressing the effectors by J774 macrophages (A) or PMNs (B). J774 macrophages or PMNs were infected at an MOI of 50:1 for 30 min at 37°C with WT [MRS40(pYV40)] *Y. enterocolitica*, the *yscN* mutant [MRS40(pMSL41)], the ΔHOPEMT mutant [MRS40(pIML421)], or the ΔHOPEMT strain overexpressing YopE (pMSL68), YopH (pMSL69), YopT (pIML279), or YopO (pMSL34). Phagocytosis percentages were determined by double-immunofluorescence assay. Phagocytosis results are given as percentages of phagocytosed bacteria relative to the total number of cell-associated bacteria (*, $P < 0.05$ compared to the ΔHOPEMT strain; $n = 6$). The values are the mean plus the standard error of the mean. For each experiment, at least 100 cells were counted. (C) Yop secretion by the *Y. enterocolitica* ΔHOPEMT mutant overexpressing the effectors. Western blot analysis (chemiluminescence detection) of proteins from culture supernatant corresponding to 2 ml of a culture of WT [MRS40(pYV40)] *Y. enterocolitica*, the *yscN* mutant [MRS40(pMSL41)], the ΔHOPEMT mutant [MRS40(pIML421)], or the ΔHOPEMT mutant overexpressing YopE (pMSL68), YopH (pMSL69), YopT (pIML279), or YopO (pMSL34) incubated for 4 h at 37°C in BHI-Ox. Immunoblot assay with rabbit anti-YopH, anti-YopT, and anti-YopO polyclonal antibodies and rat anti-YopE monoclonal antibodies (13A9). (D) Injection of the effectors by the various *Y. enterocolitica* ΔHOPEMT recombinant strains. J774 macrophages were infected at an MOI of 50:1 with WT *Y. enterocolitica* [MRS40(pYV40)], the ΔHOPEMT mutant [MRS40(pIML421)], the ΔHOPEMT mutant expressing YopE (pMSL68), the ΔHOPEMT mutant expressing YopH (pMSL69), the ΔHOPEMT mutant expressing YopT (pIML279), the ΔHOPEMT mutant expressing YopO (pMSL34), or the *yopB* mutant [MRS40(pPW401)] producing the same individual Yops. After 2 h, cytosolic fractions of J774 were prepared by Triton (0.1%) lysis and analyzed by SDS-PAGE and Western blotting with rabbit anti-YopH, anti-YopT, anti-YopO, and anti-SycE polyclonal antibodies and rat anti-YopE monoclonal antibodies (13A9). The absence of detection of SycE in the Triton-soluble fraction demonstrates that this fraction does not contain cytoplasmic proteins from *Y. enterocolitica*.

Actin cytoskeleton disruption by effectors YopE, YopH, YopT, and YopO. Since we could not see any resistance of our recombinant ΔHOPEMT clones to phagocytosis, we wondered whether they exerted their effect on the cytoskeleton. To address this question, we again used Rat-1 fibroblasts instead of phagocytes because their actin cytoskeleton is more developed than that of phagocytes. We infected Rat-1 fibroblasts for 2.5 h and stained the cytoskeleton with FITC-phalloidin (Fig. 5). The cytoskeleton of cells infected with *Y. enterocolitica* MRS40 expressing only one effector was affected less than that of cells infected with WT bacteria. Cells did not round up, remained spread, and attached to the coverslips even though the cytoskeleton was modified. Injection of YopE led to disruption

of actin stress fibers and induced accumulation of peripheral actin. YopH induced retraction of Rat-1 fibroblast and the appearance of cellular protrusions like microspikes. After injection of YopT, the actin cytoskeleton was disrupted and some aggregates of actin appeared within the Rat-1 fibroblast cells. The fibroblasts were still adherent and stayed attached after YopO injection but presented ruffle structures. Finally, after 4 h of infection, Rat-1 cells infected with these different strains rounded up and detached, as normally seen after 2.5 h of infection with WT bacteria (data not shown).

Therefore, each effector injected alone manifested its effect on the cytoskeleton. In agreement with the known diversity of targets of YopE, -H, -T, and -O, the effects were different from

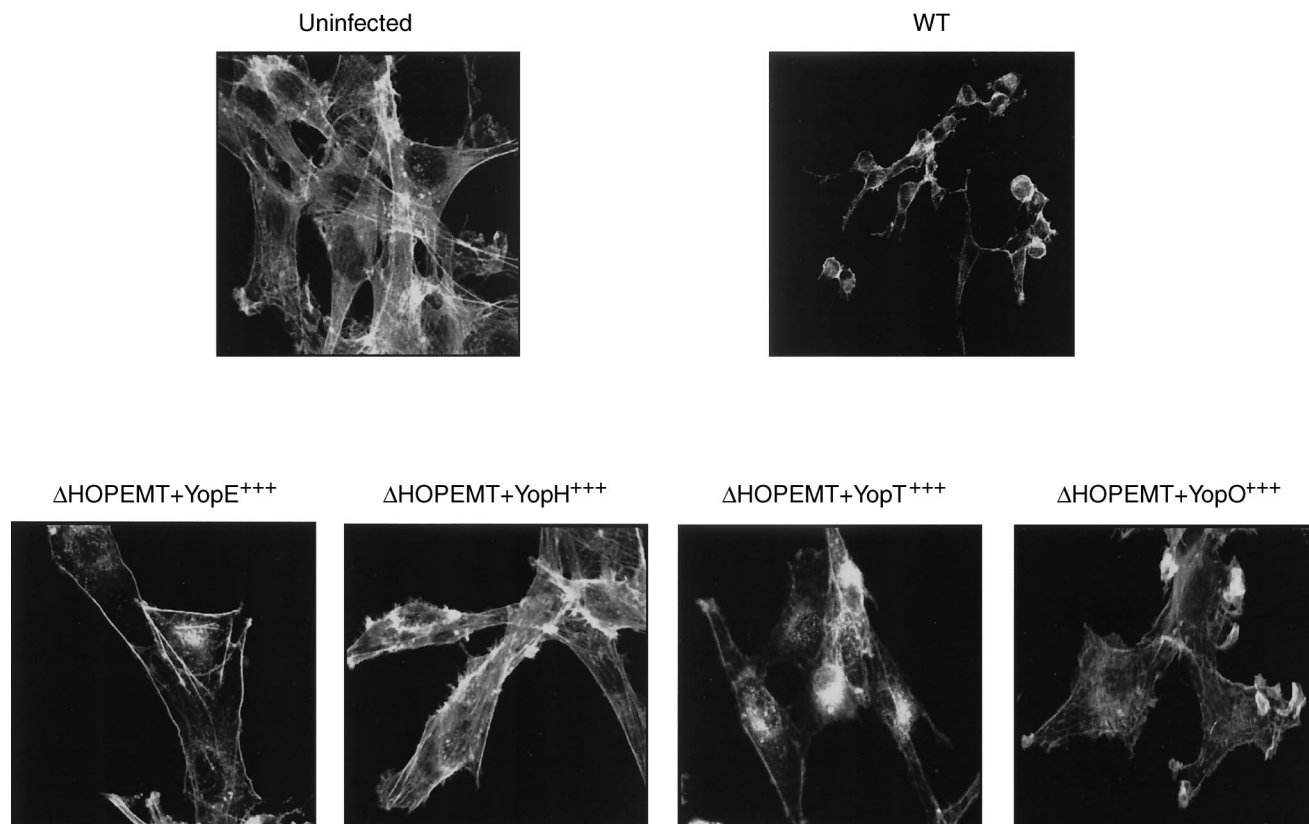


FIG. 5. Actin disruption by YopE, YopH, YopT, and YopO. Rat-1 cells were infected with *Y. enterocolitica* MRS40(pYV40) for 2 h 30 min in the presence of 10% FBS. After fixing and permeabilization of the cells, actin was stained with phalloidin-FITC. The cells were then analyzed by confocal scanning laser microscopy. Uninfected cells display elongated actin filaments (stress fibers) throughout the cells. Infection with WT *Y. enterocolitica* [MRS40(pYV40)], the Δ HOPEMT mutant [MRS40(pIML421)], and the Δ HOPEMT mutant expressing YopE (pMSL68), YopH (pMSL69), YopT (pIML279), or YopO (pMSL34) results in different modifications of the actin cytoskeleton.

each other. Thus, the lack of resistance to phagocytosis observed in Fig. 4A and B was not due to lack of Yop action. We thus concluded that YopE, -H, -T, or -O alone is not sufficient to inhibit phagocytosis.

DISCUSSION

Previous studies have unambiguously demonstrated that YopH and YopE play a major role in the defense of *Y. pseudotuberculosis* against phagocytosis (10, 25, 56, 57). In this study, we focused on *Y. enterocolitica* and addressed the question of the role of the other four known Yop effectors against opsonic or nonopsonic phagocytosis by cultured monocytes-macrophages and by PMNs. We also wanted to study whether there was redundancy in this system. Finally, we also reinvestigated the role of the two major adhesins. We first tested a set of single-knockout mutants and confirmed that, indeed, YopH and YopE are involved in resistance to phagocytosis (10, 25, 56, 57). The antiphagocytic role of YopH from *Y. pseudotuberculosis* has been documented for a long time (3, 25), as well as its role in the dephosphorylation of several focal adhesion proteins, including Fak, paxillin, FYB, p130^{cas}, and SKAPHOM (4, 7, 9, 15, 24, 28, 49, 50). More recent reports made the link between phosphorylation of paxillin and Fak and cytoskeletal assembly (15) and between focal adhesion and phagocytosis

(49, 50). It has been known for a long time that microinjection of YopE into epithelial cells leads to disruption of the actin microfilaments (57, 67). More recently, it appeared that YopE acts as a GTPase-activating protein active toward GTPases of the Rho family (g67), more precisely, toward Rac (5). Since selective inhibition of Rac abolishes ruffle formation (5), one of the engulfment steps, here again, the two kinds of observations can be combined. In this work, we confirmed that YopH contributes to the inhibition of phagocytosis of both opsonized and nonopsonized *Y. enterocolitica* bacteria by J774 mouse macrophages and by human PMNs. We also confirmed that YopE is involved in protection against nonopsonic phagocytosis. However, we did not observe any clear role of YopE against opsonic phagocytosis by J774 cells. In addition, we demonstrated that YopO and YopT are involved in the inhibition of both types of phagocytosis, even though they are probably injected in smaller amounts. Although this observation fits perfectly with the known cellular action of YopO and YopT, this role had never been reported before. It is somehow surprising that YopT plays such a clear role in defending against phagocytosis *in vitro* while it does not seem to contribute to the capacity of *Y. enterocolitica* to multiply in Peyer's patches (30). We have no explanation for this discrepancy, which is, however, not unprecedented: the same occurs with

YopP, which has very strong *in vitro* activity but a minor role *in vivo* (61). These discrepancies probably only point to the fact that the animal models have their limitations, possibly because they only allow monitoring of acute infections. YopT inactivates RhoA by inducing an unknown biochemical modification (69). Since RhoA is necessary for the actin rearrangement underlying the phagocytic event (16), this could explain the antiphagocytic role of YopT. YpkA, the homologue of YopO in *Y. pseudotuberculosis*, has been shown to localize to the inner surface of the plasma membrane, where, upon activation by actin, it works like a RhoA- and Rac-binding kinase (23, 34). As a result of this, it is expected to prevent the actin cytoskeleton rearrangement that must occur upon contact with the parasite and lead to its engulfment.

According to our results, the other two effectors YopM and YopP do not contribute to resistance to phagocytosis. This again fits quite well with the present knowledge of these effectors. YopM is a leucine-rich repeat protein to which neither a target nor a function has been assigned. It is known to migrate to the nucleus (38, 53). As for YopP, called YopJ in *Y. pestis* and *Y. pseudotuberculosis*, it inhibits transcription of proinflammatory genes by blocking the mitogen-activated protein kinase and NF- κ B pathways, presumably acting as a protease (21, 47, 58, 61). Our data thus show that of six known effectors, four play a role in defense against phagocytosis. Interestingly, they also show that single mutants are severely affected in their resistance capacity. Thus, there is no redundancy among these anti-cytoskeleton effectors and each of them has a role to play in phagocyte neutralization. This conclusion is reinforced by our complementary experiments showing that none of the four effectors alone, even when produced in larger amounts, can confer a significant level of resistance. Thus, the four effectors act in a synergistic way by blocking different key steps in the phagocytic pathway.

There was no difference in the profiles of resistance of the various single mutants to phagocytosis after opsonization either with complement or with IgG. This result is somehow surprising, as these two different opsonizations lead to phagocytosis driven by different mechanisms, in particular, by different GTPases (1, 2). The result obtained with YopT, which was only shown to affect RhoA, suggests that either RhoA is always involved or that YopT also affects other GTPases. Even more surprising is our result showing that YopE does not seem to protect at all against opsonic phagocytosis, whatever the opsonin used. This might be explained by the fact that nonopsonic phagocytosis involves a different signaling cascade than opsonic phagocytosis. YopE has been shown to inactivate Rho, Rac, and Cdc42 *in vitro* (8, 67) but only activity toward RhoA and Rac has been indirectly demonstrated *in vivo* (8). Our results thus suggest that Cdc42 contributes more to opsonic phagocytosis than to nonopsonic phagocytosis and that Rac and RhoA are involved in the nonopsonic pathway of phagocytosis. Adherence is a prerequisite for activation of the Yop virulon in contact with epithelial cells, endothelial cells, and fibroblasts (14, 68). In the absence of opsonization, the *inv yadA* double mutant has fourfold reduced adherence to phagocytes, indicating that these two bacterial adhesins can play a major role in establishing contact with phagocytes. This is in perfect agreement with previous reports by Roggenkamp et al. (54) and Ruckdeschel et al. (59) showing that YadA plays a

specific role during interaction of nonopsonized *Y. enterocolitica* and PMNs. Inv has been shown to trigger phagocytosis of nonopsonized *Y. enterocolitica* after contact with β 1 integrins, which activates actin polymerization via Cdc42Hs, its effector WASp, and the Arp2/3 complex (68). In our experiments, loss of YadA resulted in a more pronounced reduction of adherence than did loss of Inv. However, one cannot draw a conclusion from this about the relative importance of these two adhesins *in vivo* because they differ in relative abundance, depending on the experimental conditions. Indeed, Inv is produced at low temperatures while YadA is produced at 37°C and here the experiments were conducted with bacteria grown at room temperature and subsequently shifted to 37°C for 30 min. Under these conditions, both adhesins are expected to be present at the bacterial surface. In addition to being temperature regulated, *yadA* belongs to the *yop* regulon and hence is subject to feedback inhibition when the Ysc secretion channel is not functional (19). This may explain why the *yscN* mutant interacted less with phagocytes than did WT bacteria. Looking at the internalization data in the absence of opsonization, it thus appears that Inv or YadA promotes contact and internalization, which could be considered detrimental for the bacterium.

It has previously been shown that nonopsonized *Y. enterocolitica* and macrophages can interact independently of YadA and Inv (14). In agreement with these previous findings, the *inv yadA* double-mutant bacteria still interacted with phagocytes, indicating that another element, different from Inv and YadA, leads to some adherence. It could be either an unidentified bacterial adhesin or a phagocyte receptor recognizing another bacterial surface component, such as lipoglycans, mannose, or other sugar moieties. However, the Inv- and YadA-independent adherence leads to very poor internalization but our data do not reveal whether this results from poor stimulation of phagocytic activity or from excellent Yop virulon activity.

In the presence of opsonins, bacteria lacking both adhesins were efficiently associated with phagocytes and phagocytosed. This indicates that, in the presence of opsonins, bacterial adhesins do not facilitate ingestion. It is even the opposite, since YadA-deficient bacteria appear to be phagocytosed more than WT bacteria, as expected from the observations of China et al. that YadA binds factor H (17). The fact that YadA has a protective role against opsonic phagocytosis is also apparent from the effect of opsonization on phagocytosis of the *inv yadA* double-mutant bacteria: opsonization increases the percentage of phagocytosed *inv yadA* double-mutant bacteria by 45% but that of *inv yadA*⁺ mutant bacteria by only 10%. We infer that this difference reflects some protective activity of YadA against opsonization.

In conclusion, in the absence of opsonins, YopE, -H, -T, and -O act synergistically to counteract phagocytosis of *Y. enterocolitica* by macrophages and PMNs. There is no redundancy in the system since the loss of any of these Yops leads to a significant increase in phagocytosis. In the presence of opsonins, YopH, YopT, and YopO play a clear role but their synergistic action is insufficient to block phagocytosis completely. Presumably, this reduction allows sufficient escape to sustain infection. YopE does not seem to contribute to resistance to opsonic phagocytosis by macrophages. As for the adhesins, they favor phagocytosis in the absence of opsoniza-

tion. However, in the presence of opsonins, the situation is different: the adhesins do not contribute to phagocytosis, and YadA even has a protective effect.

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