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Yersinia pseudotuberculosis Inhibits Fc Receptor-Mediated Phagocytosis in J774 Cells

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Nonopsonized as well as immunoglobulin G (IgG)-opsonized *Yersinia pseudotuberculosis* resists phagocytic uptake by the macrophage-like cell line J774 by a mechanism involving the plasmid-encoded proteins Yops. The tyrosine phosphatase YopH was of great importance for the antiphagocytic effect of the bacteria. YopH-negative mutants did not induce antiphagocytosis; instead, they were readily ingested, almost to the same extent as that of the translocation mutants YopB and YopD and the plasmid-cured strain. The bacterial determinant invasin was demonstrated to mediate phagocytosis of nonopsonized bacteria by these cells. In addition to inhibiting uptake of itself, *Y. pseudotuberculosis* also interfered with the phagocytic uptake of other types of prey: J774 cells that had been exposed to virulent *Y. pseudotuberculosis* exhibited a reduced capacity to ingest IgG-opsonized yeast particles. This effect was impaired when the bacterium-phagocyte interaction occurred in the presence of gentamicin, indicating a requirement for in situ bacterial protein synthesis. The *Yersinia*-mediated antiphagocytic effect on J774 cells was reversible: after 18 h in the presence of gentamicin, the phagocytic capacity of *Yersinia*-exposed J774 cells was completely restored. Inhibition of the uptake of IgG-opsonized yeast particles was dependent on the Yops in a manner similar to that seen for blockage of *Yersinia* phagocytosis. This similarity suggests that the pathogen affected a general phagocytic mechanism. Despite a marked reduction in the capacity to ingest IgG-opsonized yeast particles, no effect was observed on the binding of the prey. Taken together, these results demonstrate that Yop-mediated antiphagocytosis by *Y. pseudotuberculosis* affects regulatory functions downstream of the phagocytic receptor and thereby extends to other types of phagocytosis.

Many bacterial pathogens infecting mammals have developed specific traits to avoid the innate and specific immune defense of the host. The three virulent species of the genus *Yersinia* have in common their capacity to resist antibacterial activities of phagocytic cells. *Yersinia pestis*, the cause of bubonic plague, and the enteropathogenic *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* exhibit different degrees of virulence but share the property of multiplying in lymphatic tissues. In contrast to many intracellular pathogens, *Y. pseudotuberculosis* replicates extracellularly in such tissues (52); this is due to the capacity of the bacteria to inhibit phagocytes from accomplishing phagocytosis (45, 46). The virulence-associated proteins participating in this phagocytic inhibition are encoded on a common 70-kb plasmid. Expression of these proteins, denoted *Yersinia* outer proteins (Yops), are regulated positively by temperature. There is also a negative control system for the expression of Yops, where the suppression is released in vitro by depletion of Ca^{2+} in the culture medium (6, 7) and in vivo by the interaction with the target cell (40, 48). Site-directed mutagenesis has identified YopH, YopE, YopM, YopK, and the recently described *Yersinia* protein kinase (YpkA) as indispensable for the virulence of *Yersinia* spp. (7, 15, 16, 33, 34). Interestingly, three of these Yops share homology with mammalian proteins. YopH, which is highly involved in phagocytic inhibition, is homologous to eukaryotic protein tyrosine phosphatases (23). It is by far the most active of all known protein tyrosine phosphatases and has also been shown to act on target cell proteins (3, 4, 61). YopE also participates in inhibition of phagocytosis and mediates contact-dependent cytotoxicity of the target cell (46, 47). Interestingly, it has recently been shown that YopE has a high degree of homology with the N-terminal end of exoenzyme S of *Pseudomonas aeruginosa* (31). YopM exhibits high homology to the α-chain of the platelet-specific receptor GpIα and prevents platelet aggregation in vitro by interacting with thrombin (33, 34). It was recently demonstrated that YpkA shares homology with eukaryotic Ser and Thr kinases. YpkA is, at present, the only prokaryotic protein kinase known to be involved in pathogenesis; the specific role played by this protein has not yet been revealed (16, 17). The role of YopK, and its counterpart in *Y. enterocolitica* (YopQ), in the infectious process is not known, but both *yopK* and *yopQ* mutants have been shown to be attenuated in mouse virulence (28, 37, 55, 56). The Yops involved in phagocytic inhibition, i.e., YopH and YopE, are assumed to function inside the phagocyte. The translocation of YopE, and presumably also YopH, to the interior of the target cell is mediated by YopB and YopD (24, 39, 47, 48, 53).

In addition to Yop-mediated resistance to phagocytosis, *Yersinia* spp. exhibit resistance to another type of innate host defense as well, namely, the complement system. Thus, complement opsonization is avoided, and thereby phagocytic ingestion is mediated by complement receptors. In *Y. pseudotuberculosis*, this property is plasmid independent (38). In *Y. enterocolitica*, on the other hand, the plasmid-encoded protein YadA is involved in preventing the deposition of complement factors (C3b and C3bi), a mechanism suggested to prevent both phagocytosis and killing by polymorphonuclear leuko-

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TABLE 1. Genotypes of the different *Y. pseudotuberculosis* strains used in the present study

Strain	Relevant genotype or phenotype	Reference
YPIII	Plasmid negative	5
YPIII(pIB102)	Wild type	6
YPIII(pIB522)	*yopE*	46
YPIII(pIB29)	*yopH*	7
YPIII(pIB251)	*yopE yopH*	46
YPIII(pIB604)	*yopB*	25
YPIII(pIB605)	*yopD*	25
YP100	Plasmid negative *invA*	49
YPI00(pIB1)	*invA*	49

cytes (9, 10, 41). However, *yadA* mutants of *Y. pseudotuberculosis* are even more virulent than the YadA-expressing wild type, indicating that YadA is less important in this strain and not a prerequisite to resist the complement system (49). In accordance with this is the finding that highly virulent *Y. pestis* does not express YadA because of a frameshift mutation in the *yadA* gene. Introduction of a functional *yadA* gene into the pathogen decreases its virulence (49).

Yersinia infections elicit a marked immune response within the host, implying that a substantial part of the infecting bacteria ought to be opsonized with immunoglobulins in the immune state (54). The aim of the present study was to investigate whether *Y. pseudotuberculosis*, in addition to neutralizing the innate immune defense, can also resist the specific immune response by preventing phagocytic uptake via Fc receptors. Another objective was to determine whether virulent *Yersinia* spp. interfere with cellular Fc receptor-mediated mechanisms for uptake in general or if such interference is restricted, in particular, to uptake of the *Yersinia* bacterium itself.

MATERIALS AND METHODS

Materials. The chemicals used and their sources were as follows: nutrient mixture Ham F-10 and fetal calf serum (GIBCO BRL, Middlesex, England), penicillin (Nord Cell, Bromma, Sweden), brain heart infusion broth (Becton Dickinson, Meylan, France), swine anti-rabbit immunoglobulin conjugates (tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate [FITC]; Dakopatts, Glostrup, Denmark), rat monoclonal anti-mouse FcγII/III receptor antibody 2.4G2 (immunoglobulin G2b [IgG2b]) (57, 58) and rat monoclonal anti-Mac 3 antibody M3/84 (IgG1) (42) (Pharmingen, San Diego, Calif.), gentamicin and fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.), Airvol (Air Products and Chemicals, Utrecht, The Netherlands), and Citifluor (Citifluor Limited, London, United Kingdom).

Cell culture and bacterial growth. The mouse macrophage-like cell line J774A.1 was grown in nutrient mixture Ham F-10 supplemented with 10% fetal calf serum and 100 U of penicillin per ml; culturing was performed at 37°C in a humidified atmosphere with 5% CO_2. Two days before infection with bacteria, experimental cells were seeded onto coverslips (11-mm diameter; 5×10^4 to 10×10^4 cells per cm^2) in a 24-well plate. The *Y. pseudotuberculosis* strains used in this study are listed in Table 1. For maximal expression of Yop proteins, bacteria were cultured at 26°C in brain heart infusion broth supplemented with 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] and 20 mM $MgCl_2$ on a rotary shaker overnight. These cultures were diluted to 10^8 bacteria per ml (optical density at 550 nm, 0.1) and incubated as described for an additional 1 h at 26°C. Thereafter, the bacterial cultures were transferred to 37°C and incubated for an additional 2 h.

Bacterial infection. Cells grown on coverslips were washed and covered with cell culture medium supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) but lacking penicillin and were then incubated at 37°C for 2 to 3 h in a humidified atmosphere with 5% CO_2. A bacterial suspension which had been pregrown for maximal Yop expression was washed and resuspended to 10^8 bacteria per ml in the same medium as that described above. The 24-well plates with the J774 cells were preincubated for 10 min in a 37°C water bath, after which bacteria were added to the wells (5×10^6 bacteria per cm^2). The cells were infected for various periods of time (see figure legends), and the infection was terminated by transferring the culture plates to ice.

Detection of uptake of bacteria by J774 cells. Bacteria associated with the target cells in intra- and extracellular locations were distinguished essentially as described previously (27, 45). To stain extracellularly located bacteria, the coverslips with infected cells were washed and then covered with rabbit anti-*Yersinia* antiserum (diluted 1:500) (45) and kept on ice for 30 min. This antiserum was prepared as described by Doyle et al. (12) with strain YPIII(pIB1). Thereafter, excess antiserum was removed by four washes in phosphate-buffered saline (PBS), after which the coverslips were air dried and then fixed in ice-cold methanol for 90 s. The coverslips were then dried again and subsequently covered with tetramethyl rhodamine isothiocyanate-conjugated swine anti-rabbit immunoglobulins (12 μg/ml) and incubated for 20 min at 37°C; this was followed by four washes in PBS. In experiments with IgG-opsonized bacteria, this incubation was performed for 30 min at 4°C before fixation. To stain all bacteria actually associated with the J774 cells, the coverslips were again covered with anti-*Yersinia* antiserum and incubated for 1 h at 37°C and then washed four times in PBS, overlaid with FITC-conjugated swine anti-rabbit immunoglobulins (12 μg/ml), and incubated for 20 min at 37°C. Finally, after being rinsed four times in PBS, the coverslips were mounted by placing them upside down onto a drop of mounting medium on a glass slide; the mounting medium consisted of 20% Airvol and 4% Citifluor in 20 mM Tris (pH 8.5). The next day, the specimens were examined in a fluorescence microscope (Axioscope; Carl Zeiss, Oberkochen, Germany) equipped with a Plan-apochromate 63×/1.40 oil immersion objective. For each cell, the number of extracellularly located bacteria was detected by excitation at 530 to 585 nm, and the total number of cell-associated bacteria was detected by excitation at 450 to 490 nm; in each experiment, this was done for at least 100 cells per coverslip in randomly selected fields.

FITC labeling of yeast particles. FITC labeling of yeast particles was performed by the method of Hed and Stendahl (26). Heat-killed *Saccharomyces cerevisiae* organisms (10^9/ml) were incubated in 0.5% carbonate buffer (pH 9.5), containing FITC (0.1 mg/ml), for 30 min at 37°C. Thereafter, the FITC-conjugated yeast particles were washed four times in Krebs-Ringer phosphate buffer (pH 7.3) and kept at −20°C until used.

Opsonization with IgG. To IgG opsonize bacteria grown for maximal expression of Yops, anti-*Yersinia* antiserum (diluted 1:250) was added during the last 30 min of incubation at 37°C. Opsonized *Y. pseudotuberculosis* was surface characterized by staining with FITC-conjugated anti-rabbit IgG. FITC-labeled yeast particles were opsonized by suspending them in Krebs-Ringer phosphate buffer (5×10^7/ml) and then incubating them for 30 min at 37°C in the presence of anti-yeast IgG (20 μg/ml) and 20% heat-inactivated (45 min at 56°C) human serum. The inactivated serum was included to cover yeast surface structures that might function as phagocytic ligands. Thereafter, the particles were washed three times in Krebs-Ringer phosphate buffer and resuspended to the appropriate concentration. Nonopsonized yeast particles were obtained by incubating them as described above but without rabbit anti-yeast IgG.

Determination of phagocytosis of yeast particles. Phagocytosis of FITC-labeled yeast particles by J774 cells was monitored by a fluorescence quenching method (26). The yeast particles were added at a ratio of 15:1 to J774 cells grown on coverslips. The cells were allowed to phagocytose for 30 min at 37°C; phagocytosis was then interrupted by transferring the coverslips to ice. The degree of phagocytosis was determined in a fluorescence microscope (Zeiss Axioscope) equipped with a Plan-apochromate 63×/1.40 oil immersion objective. FITC-labeled yeast particles were detected with an excitation light of 450 to 490 nm. The fluorescence of the extracellularly located particles was quenched by adding a drop of trypan blue (1 mg/ml; pH 4.4) to the coverslip. In each experiment, at least 100 cells per coverslip in randomly selected fields were studied to determine the number of yeast particles attached to the surfaces and located within the target cells.

RESULTS

Phagocytosis of nonopsonized *Y. pseudotuberculosis* by J774 cells. *Y. pseudotuberculosis* has been shown previously to resist the phagocytic uptake by mouse peritoneal macrophages via a plasmid-encoded mechanism (45, 46). Cells of the macrophage-like line J774 were affected by *Y. pseudotuberculosis* in a similar manner (Fig. 1). The virulence plasmid-containing wild-type strain YPIII(pIB102) markedly inhibited the phagocytic capacity of the J774 cells. Of all the bacteria that became associated with the cells, 75% remained extracellularly bound; for the plasmid-cured strain, only 19% was located outside. In agreement with earlier studies (45, 46) with peritoneal macrophages, *Yersinia*-mediated antiphagocytosis is also dependent on the virulence plasmid when J774 cells are used. The ability of the *yopH* deletion mutant [YPIII(pIB29)] to inhibit phagocytosis was greatly reduced (Fig. 1); further reduction of the antiphagocytic capacity was seen for the *yopE yopH* double mutant [YPIII(pIB251)]. In addition, the mutants lacking

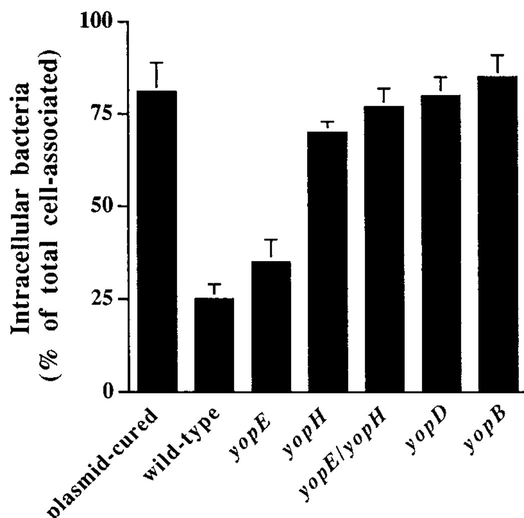

FIG. 1. Phagocytosis of nonopsonized *Y. pseudotuberculosis* by J774 cells. The *Yersinia* strains were exposed to J774 cells at a calculated bacterium/cell ratio of 20:1 for 30 min at 37°C. Determination of cellular association and discrimination between intra- and extracellularly located bacteria were performed as described in Materials and Methods. The data given represent means ± standard errors of the means of four to nine separate experiments and are expressed as percentages of J774 cell-associated bacteria that were located intracellularly. The total numbers of cell-associated bacteria were as follows: 8.2 ± 3.4 (n = 9) for the wild type; 6.8 ± 1.9 (n = 4) for *yopE*; 7.8 ± 2.5 (n = 7) for *yopH*; 8.8 ± 3.5 (n = 5) for *yopE yopH*; 7.8 ± 3.2 (n = 5) for *yopD*; 5.9 ± 3.1 (n = 4) for *yopB*.

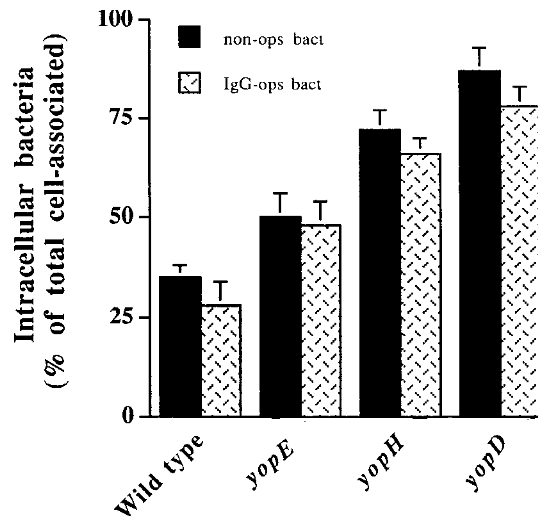

FIG. 2. Phagocytosis of IgG-opsonized *Y. pseudotuberculosis* by J774 cells. Nonopsonized or IgG-opsonized yersiniae were exposed to J774 cells at a calculated bacterium/cell ratio of 20:1 for 30 min at 37°C. Determination of cellular association and discrimination between intra- and extracellularly located bacteria were performed as described in Materials and Methods. The data given represent means ± standard errors of the means of five to six separate experiments and are expressed as percentages of J774 cell-associated bacteria that were located intracellularly.

YopB or YopD [YPIII(pIB604) and YPIII(pIB605)], proteins involved in translocation of YopE and, possibly, YopH into the target cell, were phagocytosed by J774 cells (Fig. 1). The level of phagocytosis of these mutants was of the same magnitude as that noted for the plasmid-cured strain (YPIII). Despite differences in phagocytic resistance, the total numbers of bacteria associated with the J774 cells were similar for the different strains (see legend to Fig. 1).

Phagocytosis of IgG-opsonized *Y. pseudotuberculosis* by J774 cells. Experiments with IgG-opsonized *Y. pseudotuberculosis* were performed to elucidate whether the antiphagocytic effect is restricted to uptake via a macrophage extracellular structure(s) mediating phagocytosis of nonopsonized bacteria or whether the effect is more general, i.e., includes other types of endocytosis. As for nonopsonized *Y. pseudotuberculosis*, the wild-type strain coated with rabbit anti-*Yersinia* antibodies blocked phagocytic uptake of itself (Fig. 2). IgG-opsonized *yopE*, *yopH*, and *yopD* mutant strains were also tested, and it was found that phagocytosis of these resembled phagocytosis of the corresponding nonopsonized mutants (Fig. 2). A strong staining of the IgG-opsonized bacteria with FITC-conjugated anti-IgGs revealed the presence of IgG on the bacterial surface (data not shown). However, blocking experiments with the anti-Fc receptor antibody 2.4G2 (57, 58) did not show any reduction of the uptake of the plasmid-cured strain opsonized with IgG (Fig. 3), suggesting the presence of an additional structure on the bacteria recognized by another type of phagocytic receptor on the J774 cell. The *Yersinia* determinant invasin, which binds to β_1-integrins, has been shown to induce another type of cellular uptake, namely, bacterial invasion into epithelial cells (29, 30). Plasmid-cured *invA* mutants of *Y. pseudotuberculosis* (YP100) were shown to be poorly ingested by these cells. If this strain was opsonized with IgG, ingestion by J774 cells increased to the same extent as it did for the invasin-expressing strain. This uptake was blocked when cells were preincubated with the anti-Fc receptor antibody 2.4G2 (Fig. 3). This result shows a role for invasin as a *Y. pseudotuberculosis* determinant mediating phagocytosis. Plasmid-containing *invA* mutants [YP100(pIB1)] remained extracellular as a result of phagocytic inhibition mediated by the plasmid-encoded Yops. IgG opsonization did not affect the antiphagocytic property of this strain. In fact, a more-pronounced antiphagocytic effect was seen with the opsonized bacteria (Fig. 3), clearly demonstrating the capacity of *Y. pseudotuberculosis*

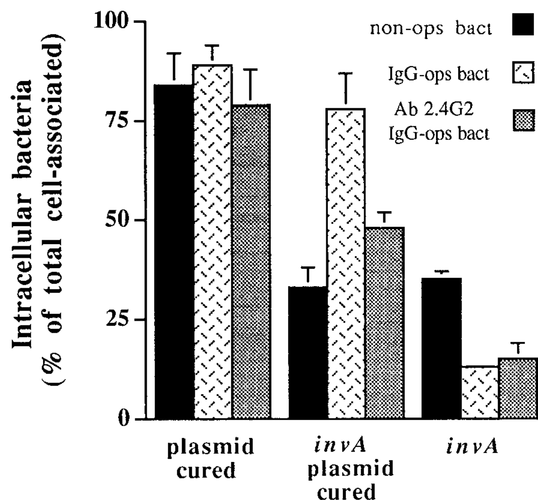

FIG. 3. Effect of Fc receptor blockage on uptake of IgG-opsonized *Y. pseudotuberculosis*. J774 cells were incubated in culture medium with or without antibody 2.4G2 (30 μg/ml) for 1 h at 37°C with 5% CO_2 prior to exposure to nonopsonized or IgG-opsonized yersiniae at a calculated bacterium/cell ratio of 10:1 for 30 min. Determination of cellular association and discrimination between intra- and extracellularly located bacteria were performed as described in Materials and Methods. The data given represent means ± standard errors of the means of four separate experiments and are expressed as percentages of J774 cell-associated bacteria that were located intracellularly.

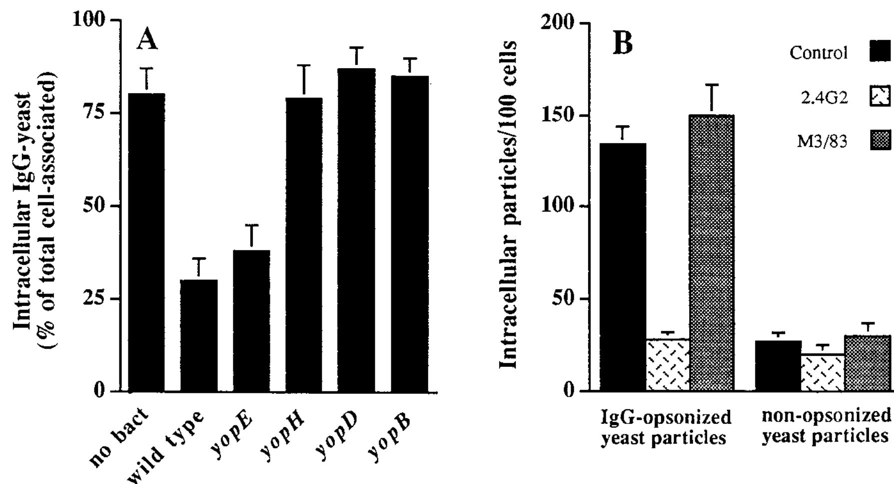

FIG. 4. Effect of *Y. pseudotuberculosis* on uptake of IgG-opsonized yeast particles by J774 cells. (A) Cells were preexposed to the *Yersinia* strains at a calculated bacterium/cell ratio of 80:1 for 30 min at 37°C and then exposed to IgG-opsonized yeast particles at a calculated yeast particle/cell ratio of 15:1. The cells were allowed to phagocytose for 30 min, and the number of particles ingested was then determined as described in Materials and Methods. The data given represent means ± standard errors of the means of four separate experiments and are expressed as percentages of J774 cell-associated IgG-opsonized yeast particles that were located intracellularly. (B) Cells were incubated in the presence or absence of antibody 2.4G2 (30 μg/ml) or M3/84 (30 μg/ml) for 1 h at 37°C with 5% CO_2 prior to exposure to nonopsonized or IgG-opsonized yeast particles at a calculated yeast particle/cell ratio of 5:1. The cells were allowed to phagocytose for 30 min, and the number of particles ingested was then determined as described in Materials and Methods. The data given represent means ± standard errors of the means of four separate experiments and are expressed as number of ingested particles per 100 cells.

to also inhibit its own uptake when it was mediated by Fc receptors.

Effect of *Y. pseudotuberculosis* on ingestion of IgG-opsonized yeast particles by J774 cells. To further clarify the nature of the phagocytic inhibition, the ability of *Y. pseudotuberculosis* to inhibit uptake of a nonrelated prey was investigated. J774 cells were preincubated with wild-type *Y. pseudotuberculosis* or mutants thereof (indicated in Fig. 4A) 30 min prior to exposure to IgG-opsonized yeast particles. Those cells preexposed to the wild-type strain showed a markedly reduced capacity to ingest the IgG-opsonized particles, i.e., only 20% of all cell-associated yeast particles were actually found within the cells. In comparison, 80% of the associated yeast particles were ingested by J774 cells that were not preexposed to bacteria. Cells preincubated with *yopH*, *yopB*, and *yopD* mutants showed no reduction in their ability to ingest IgG-opsonized yeast particles. In fact, the effect of *Y. pseudotuberculosis* on phagocytosis of IgG-opsonized yeast particles paralleled that of the uptake of the bacterium itself (compare the effects of the different strains illustrated in Fig. 1 and 4A). Control experiments performed to elucidate the specificity of the interaction of the opsonized particles with Fc receptors on the J774 cells showed that nonopsonized yeast particles were not ingested by these cells, nor were they when the cells were pretreated with antibody 2.4G2 (Fig. 4B). M3/84, an antibody recognizing the macrophage surface antigen Mac-3 (42), was included as a control of the unspecific effect of surface-bound IgG. Pretreatment with this antibody did not affect the uptake of IgG-opsonized bacteria (Fig. 4B). Immunofluorescent staining with FITC-conjugated rabbit anti-rat IgGs revealed the presence of M3/84 on the bacterial surface (data not shown). Compared with nontreated control cells, M3/84 did not reduce cellular binding of the particles; total association measurements were 163 ± 10 particles per 100 nontreated cells and 162 ± 30 particles per 100 M3/84-treated cells. 2.4G2 effectively blocked the association of IgG-opsonized particles to J774 cells (total association was 28 ± 6 particles per 100 cells).

Since the bacterium/cell ratio used in the experiments with IgG-opsonized particles was relatively high (80 added bacteria per cell), titrations were performed to determine the bacterial concentration required for inhibition of Fc receptor-mediated phagocytosis. It was necessary to add only 10 bacteria per cell to cause an antiphagocytic effect in J774 cells (Fig. 5). Immunofluorescent staining of bacteria associated with the target cells revealed that the actual bacterium/cell ratio needed for this inhibition was only 4.2:1 (Fig. 5). At a calculated number of 80 added bacteria per cell (13.6 ± 4 bacteria actually associated with each cell) (Fig. 5), the phagocytic capacity of the J774 cells was reduced by 69% ± 11% (Fig. 5) without affecting cellular viability (revealed by trypan blue staining; data not shown). This bacterial concentration was chosen for studies of the kinetics for induction of phagocytic inhibition. After 5 min of preincubation with the bacteria, the macrophage phagocytic capacity (determined after 30 min of exposure to opsonized yeast particles) was reduced by 20% ± 7% (Fig. 6); at that time point, only 7.3 ± 0.7 bacteria were associated with the cells (Table 2). After 20 min of preexposure to bacteria, cellular uptake of IgG-opsonized yeast particles was reduced by 53% ± 13%. The time scale for preexposure to bacteria (Fig. 6 and Table 2) does not necessarily show the length of time the bacteria were actually associated with the surface of the target cells. It should also be noted that this time scale indicates the times of bacterial preexposure and that the bacteria were also present during the 30 min of interaction with the yeast particles. Preexposure to the wild-type strain did not impair the capacity of J774 cells to bind IgG-opsonized yeast particles; instead, a slight increase in the number of IgG-opsonized yeast particles associated with the cell surface was observed (Table 2). This might mean that the presence of bacteria preactivates the target cells, making them more effective in binding the prey.

Dependency of *Y. pseudotuberculosis* protein synthesis for phagocytic inhibition. It has been suggested recently that bacterial protein synthesis is a prerequisite of YopE-mediated cytotoxicity (46). In accordance with this finding, the presence of gentamicin during preincubation of J774 cells with wild-type *Y. pseudotuberculosis* impaired the bacterium-mediated phagocytic inhibition (Fig. 6). However, a slight reduction of the

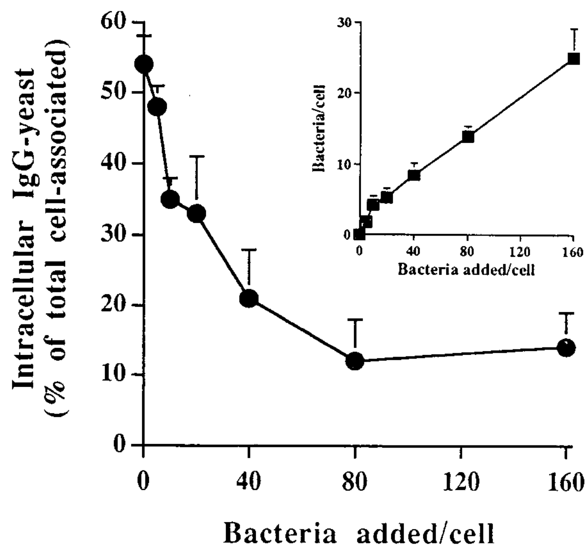

FIG. 5. Effect of increasing concentrations of *Y. pseudotuberculosis* on uptake of IgG-opsonized yeast particles by J774 cells. Cells were preexposed to wild-type *Y. pseudotuberculosis* at the calculated bacterium/cell ratios indicated for 30 min at 37°C. Thereafter, they were exposed to IgG-opsonized yeast particles at a calculated yeast particle/cell ratio of 15:1. The cells were allowed to phagocytose for 30 min, and the number of particles ingested was then determined as described in Materials and Methods. The data given represent means ± standard errors of the means of five separate experiments and are expressed as percentages of J774 cell-associated IgG-opsonized yeast particles that were located intracellularly. The inset shows the actual number of bacteria associated per cell after 30 min of incubation with the indicated amount of added bacteria. Cells were treated with bacteria as described above and then gently washed and fixed for 90 s in methanol. The total number of cell-associated bacteria was determined by fluorescent staining as described in Materials and Methods. The data given are means ± standard errors of the means of five separate experiment and are expressed as the total number of bacteria that was associated with one cell.

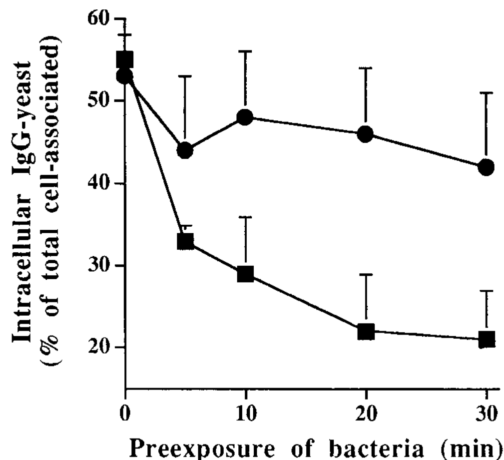

FIG. 6. Effect of gentamicin on the ability of *Y. pseudotuberculosis* to mediate phagocytic inhibition and the effect of different times of bacterial preexposure on uptake of IgG-opsonized yeast particles by J774 cells. Cells were preexposed, for the indicated periods of time, to wild-type yersiniae in the absence of gentamicin (■) or in the presence of 100 μg of gentamicin per ml ( ) at the calculated bacterium/cell ratio of 80:1. Thereafter, the cells were exposed to IgG-opsonized yeast particles at a calculated yeast particle/cell ratio of 15:1 and allowed to phagocytose for 30 min. The number of particles ingested was then determined as described in Materials and Methods. The data given represent means ± standard errors of the means of four separate experiments and are expressed as percentages of J774 cell-associated IgG-opsonized yeast particles that were located intracellularly.

phagocytic capacity was still observed. This could have been due to the bacterial growth conditions used in this experiment, i.e., the bacteria were precultured in the absence of Ca^{2+} at 37°C to obtain maximal Yop expression. These culturing conditions induce high levels of expression and subsequent secretion of Yops into the culture medium (13). For comparison, similar experiments were performed in which the bacteria were precultured in the presence of Ca^{2+}. Under such growing conditions, expression and translocation of Yops are not induced until the bacteria interact with the target cells (48). This pretreatment resulted in an even more pronounced blocking effect of gentamicin on the antiphagocytosis mediated by *Y. pseudotuberculosis* (not shown). Cells whose phagocytic capacity was reduced by 55% ± 9% by preexposure to the wild-type strain could, after 18 h in the presence of gentamicin (10 μg/ml), ingest IgG-opsonized yeast particles to the same extent as that of non-bacterium-treated control cells (Fig. 7). Thus, the antiphagocytic effect was reversible and did not primarily affect the viability of the cells. The recovery of the cells was time dependent, with a 50% recovery after 8 to 10 h (data not shown) and full recovery after 18 h.

DISCUSSION

The present study demonstrates that phagocytosis of *Y. pseudotuberculosis* by J774 cells can be blocked by the bacteria themselves. The bacterium is also able to block its own phagocytosis when ingestion occurs via activation of Fc receptors. This indicates that in J774 cells, the target for the Yops is downstream from the phagocytic receptor and participating in the signaling pathway mediating phagocytosis. Opsonization with IgG very effectively enhances the host defense by facilitating macrophage recognition and phagocytosis of invading microorganisms. The demonstrated virulence property of *Y. pseudotuberculosis* should make this pathogen highly resistant to the host immune defense and could also provide an explanation to the pathogenicity of the bacteria, i.e., why the microbe can survive in the phagocyte-rich lymph nodes despite the presence of substantial amounts of *Yersinia*-directed antibodies. Wild-type *Y. pseudotuberculosis* not only inhibits phagocytosis of itself by J774 cells (45, 46; this study) but also impairs the capacity of these cells to ingest other kinds of prey. For example, IgG-opsonized yeast particles, which, under normal conditions, are readily ingested by J774 cells (20), remain extracellularly bound on the macrophages preexposed to virulent *Y. pseudotuberculosis*. The presence of surface-bound *Y. pseudotuberculosis* did not reduce binding of IgG-opsonized yeast particles to the cells; instead, there was a slight increase in cell-associated IgG-opsonized yeast particles. This excludes the possibility that *Y. pseudotuberculosis* interacts directly with Fc receptors. It is obvious that the Yops are involved in the inhibition of phagocytosis of IgG-opsonized yeast particles

TABLE 2. Quantitation of cell-associated bacteria by immunofluorescent staining[a]

Time of preexposure to bacteria (min)	No. of bacteria/cell	No. of yeast particles/cell
0		8.1 ± 1.1
5	7.3 ± 0.7	7.9 ± 0.9
10	10.2 ± 1.2	8.5 ± 1.5
20	13.8 ± 2.4	9.6 ± 1.7
30	18.4 ± 3.7	10.3 ± 2.2

[a] Means ± standard errors of the means of four to seven experiments. The numbers of bacteria and IgG-opsonized yeast particles associated with each cell were determined at 0 and 30 min, respectively. See text for details.

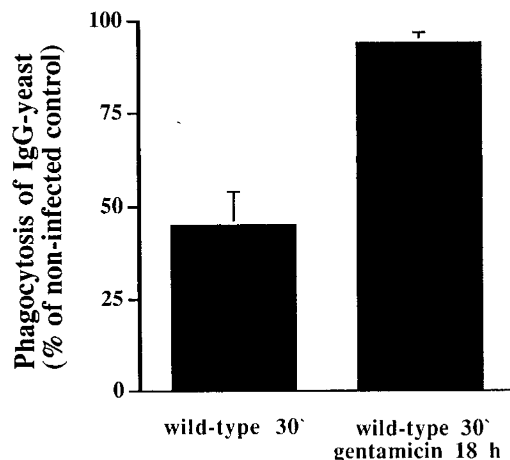

FIG. 7. Recovery of phagocytic capacity of *Y. pseudotuberculosis*-treated J774 cells. Cells were preexposed to the *Y. pseudotuberculosis* strains indicated at a calculated bacterium/cell ratio of 80:1 for 30 min at 37°C. The preexposed J774 cells were then divided into two portions. One portion was exposed to IgG-opsonized yeast particles at a calculated yeast particle/cell ratio of 15:1 and allowed to phagocytose for 30 min, after which the number of ingested particles was determined as described in Materials and Methods. The other portion was washed and supplemented with gentamicin (10 μg/ml) and incubated for 18 h at 37°C in a humidified atmosphere with 5% CO_2; phagocytosis of IgG-opsonized yeast particles was then allowed to proceed and determined as described above. The data given represent means ± standard errors of the means of four separate experiments and are expressed as percentages of controls (percentages of cell-associated IgG-opsonized particles that were located intracellularly in cells not treated with *Y. pseudotuberculosis*). The percentages of intracellularly located IgG-opsonized particles in the absence of bacteria were 86.8% ± 3.2% (left) and 87.7% ± 4.5% (right).

since cellular uptake of these particles after preexposure to the wild type or the different mutant strains resembles the uptake of the bacterium itself. Only a short time of preexposure and a small number of attached bacteria were required to induce this effect, which indicates a very effective blocking mechanism with a key target(s) being part of either the general molecular machinery for uptake or a molecule(s) involved in the regulation of that machinery.

In contrast to what has been reported previously for mouse peritoneal macrophages (45, 46), antiphagocytosis in J774 cells is mediated primarily via YopH activity. YopE, which is involved in the inhibition of mouse peritoneal macrophages, is also, but to a lesser degree, involved in the inhibition of J774 cells. A possible explanation for these differing YopE sensitivities between different types of macrophages could be differences in cell maturation or the degree of activation. The importance of the tyrosine phosphatase YopH for immediate phagocytic inhibition implies that the primary target in phagocytic inhibition is a tyrosine-phosphorylated protein. This target could be (i) a constantly phosphorylated protein that is an indispensable part of the cellular uptake machinery or (ii) a protein that is tyrosine phosphorylated upon phagocytic activation and subsequently dephosphorylated by YopH, whereupon an intracellular phagocytic signal is interrupted. The present finding that YopH is an important inhibitor of Fc receptor-mediated phagocytosis is in accordance with the recent finding that uptake via this receptor in macrophages is highly dependent upon tyrosine kinase activity (21). The Fc receptor-associated kinase activity involves phosphorylations of the following tyrosine kinase substrates: paxillin, $p72^{syk}$, and the γ subunit of the receptor (22). Moreover, several reports have shown that ligation of this receptor, either with IgGs or by cross-linking, is associated with tyrosine kinase activity (11, 18, 19, 22, 50). During Fc receptor-mediated phagocytosis, tyrosine-phosphorylated proteins assemble at the site of phagocytic invagination. In other types of cells, tyrosine-phosphorylated proteins are associated with focal adhesion plaques, where they participate in the contact between adhesion receptors (integrins) and the actin cytoskeleton (60). The assembly of tyrosine-phosphorylated proteins at the phagocytic site might represent the formation of contact bridges of this type. If that is true, YopH can act on these bridges and thereby break the route for outside-in signaling that leads to the cytoskeleton-dependent phagocytosis. The observation that only ingestion, and not attachment, of the particles was affected by YopH is in accordance with studies of cellular spreading, where focal adhesion formation and tyrosine phosphorylation are prerequisites of the cytoskeleton-dependent spreading but not of the initial attachment of the receptor to the underlying substrate (60). Also of relevance in this context is the suggested involvement of tyrosine kinase activity in other cellular cytoskeleton-dependent processes, e.g., bacterium-induced phagocytosis in epithelial cells, phagocytosis of yeast particles, chemotaxis, motility, receptor internalization, neurite outgrowth, and invasion into matrix by transformed cells (1, 2, 32, 35, 36, 43, 44, 51, 59).

Bacterial expression of invasin is shown to be necessary for uptake of nonopsonized bacteria, indicating a property of this molecule to stimulate an uptake mechanism in phagocytic cells also. Opsonization with IgG did not enhance phagocytosis, i.e., nonopsonized bacteria are ingested to the same extent as IgG-opsonized bacteria, suggesting invasin to be very effective for this purpose and that the degree of phagocytosis seen for the individual receptors is maximal for these experimental conditions. This also provides an explanation for the previous finding that an *invA* mutant of *Y. pseudotuberculosis* in a certain genetic background is profoundly more virulent than the invasin-expressing strain (49). This mutant lacks the ligand necessary for recognition by phagocytic receptors on the macrophage, thereby enabling it to effectively avoid phagocytosis. The phagocytic receptor that mediates uptake of nonopsonized yersiniae is not known, but our results favor the previous suggested involvement of invasin-integrin interactions (29, 30). Invasin binds to different variants of β_1-integrin, a receptor suggested to mediate invasion of yersiniae into epithelial cells (30). Whether this receptor, which normally functions as an adhesion receptor, also acts as a phagocytic receptor on phagocytes or whether there is another receptor involved is still controversial (8).

The present study shows that *Y. pseudotuberculosis* affects a general phagocytic effector(s), which suggests that phagocytic pathways with different starting points converge to form a common route that leads to actin rearrangement favoring particle uptake. One example of this is the coupling between the Fc receptors, FcγRII/III, and the β_2-integrin complement receptor type three. These are two major phagocytic receptors that are linked in such a way that they are coupled and act in synergy to achieve effective engulfment and subsequent killing of the prey; for instance, activation of complement receptor type three leads to FcγRII association with the actin cytoskeleton (62).

Yop proteins are not found in the extracellular space during infection, and an in vitro-obtained, Yop-containing supernatant has no antiphagocytic or cytotoxic effect on the target cell (47). Expression of these proteins in vivo is suggested to be induced by binding of the bacteria to an unknown structure(s) on the surface of the target cell, followed by a polarization of the Yops in the bacteria, and, thereafter, translocation of YopE, and probably also YopH, into the target cell (14, 39,

48). This assumption is supported by the notion that both of the translocation mutants (*yopB* and *yopD*) are unable to prevent phagocytosis, although they are able to express and secrete active YopE and YopH. This also implies that bacterial protein synthesis is a prerequisite for the antiphagocytic effect of yersiniae. The hypothesis of cell contact-mediated induction of Yop proteins is further strengthened by the present findings that the presence of gentamicin nearly abolishes the ability of *Y. pseudotuberculosis* to inhibit phagocytosis of IgG-opsonized yeast particles. In addition, like YopE-mediated cytotoxicity in HeLa cells (46), *Yersinia*-mediated antiphagocytosis is a reversible mechanism which can be overcome by treatment with gentamicin. This suggests that the Yop-mediated effects are not directed primarily against cellular viability; instead, specific antimicrobial mechanisms are affected initially.

Taken together, the present findings demonstrate that *Yersinia*-mediated antiphagocytosis includes uptake via Fc receptors. This ability renders the pathogen capable of resisting the specific host defense. Moreover, *Yersinia* spp. induce general inhibition of phagocytosis, where YopH plays an important role, probably by affecting a target molecule(s) acting downstream of the phagocytic receptor.

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