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Differential Contribution of *Yersinia enterocolitica* Virulence Factors to Evasion of Microbicidal Action of Neutrophils

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The differential contribution of the virulence factors invasin, protein tyrosine phosphatase (YopH), cytotoxin (YopE), and adhesin (YadA) of *Yersinia enterocolitica* to evasion of the antibacterial activities of polymorphonuclear leukocytes (PMNs) (oxidative burst, phagocytosis, killing) was analyzed. We constructed virulence gene knockout mutants and a novel two-plasmid system allowing production and secretion of individual virulence factors. Wild-type *Y. enterocolitica* WA-314 harboring the virulence plasmid pYVO8 resisted phagocytosis and killing by PMNs. Moreover, strain WA-314 was able to inhibit the neutrophil oxidative burst upon stimulation with opsonized zymosan independently on preincubation with normal human serum or YadA-specific serum. These phenotypic properties of strain WA-314 were differentially affected when mutants impaired in YadA production or Yop secretion were used. A more detailed analysis revealed that YopH plays the dominant role in suppression of the antibacterial action of PMNs without damaging the cells. The YopH suppressing effect could be enhanced by coproduction of YopE and YadA. The contribution of YadA is attributed to the adhesin function promoting interaction with PMNs under both opsonizing and nonopsonizing conditions. In contrast, invasin seems to mediate only opsonin-independent interaction with PMNs. Taken together, our results demonstrate that YopH, YopE, and YadA act in concert towards neutrophil attack to enable extracellular survival of *Y. enterocolitica* in host tissue.

Yersinia enterocolitica is an enteric pathogen causing a variety of gastrointestinal and systemic syndromes, including enterocolitis, terminal ileitis, mesenteric lymphadenitis, and septicemia (7). Infection is initiated when ingested *Y. enterocolitica* cells penetrate Peyer's patches and proliferate within the lymphoid tissue of the small intestine (17). From there, bacteria may be disseminated throughout the body. There is evidence that the chromosome-encoded outer membrane protein invasin (Inv) is required for the initial entry process (32). Inv mediates binding to multiple β_1 integrin receptors of mammalian cells and subsequently promotes internalization of the microorganism (25). For survival in host tissue, virulent yersiniae have developed strategies to resist the nonspecific immune defense of the host. This capability depends on the presence of a 70-kb virulence plasmid (pYV) that encodes a set of secreted proteins, termed Yops (*Yersinia* outer proteins) (20, 47), and the adhesin YadA. YadA forms a fibrillous matrix at the surface of the pathogen (26) and confers multiple virulence properties like adherence to epithelial cells (21) and to extracellular matrix proteins (9, 44, 45, 48). Moreover, YadA counteracts opsonization of bacteria with complement (6, 34) and mediates resistance to human serum (1, 22). Expression of YadA and Yops is temperature dependent, and synthesis occurs during growth at 37°C (27, 47). Individual cytoplasmatic chaperones are necessary for the secretion of Yops, i.e., SycE for YopE and SycH for YopH (52, 53).

YopE and YopH of *Y. pseudotuberculosis* have antiphagocytic activities upon macrophages (39, 40). YopE and, presumably, also YopH are translocated into mammalian cells after contact (polarized transfer) (41, 46). YopE causes disruption of actin microfilaments and thus mediates cytotoxicity (40, 41). YopH has been identified as a protein phosphotyrosine phosphatase (15) and probably interferes with the host cell signalling pathways involved in phagocytosis of bacteria by macrophages (3, 11, 39). Recent investigations gave evidence that YopH also plays role in inhibition of the Fc receptor-mediated oxidative burst of macrophages (2). However, another report demonstrated that YopH has no impact on macrophage respiratory burst inhibition upon stimulation with nonopsonized zymosan (14, 18).

Concerning the interaction of *Y. enterocolitica* with polymorphonuclear leukocytes (PMNs), the YadA protein is supposed to be of major importance in preventing phagocytosis of *Y. enterocolitica* in the presence of complement (5). On the other hand, recent investigations could not demonstrate a contribution of YadA to phagocytosis inhibition in the presence of *Y. enterocolitica* immune serum but attributed this inhibitory function to the Yop proteins (50). Thus, the role of the distinct virulence factors of *Y. enterocolitica* in interaction with PMNs is still confusing.

The aim of the present study was to reanalyze the response of PMNs to *Y. enterocolitica* in the presence and absence of serum opsonins and to determine the impact of distinct *Yersinia* virulence factors (YadA, YopH, YopE, and Inv) on neutrophil phagocytosis, killing, and oxidative burst inhibition.

MATERIALS AND METHODS

Reagents. Luminol, zymosan, phorbol myristate acetate, superoxide dismutase, horseradish peroxidase, catalase, sodium azide, propidium iodide, Tergitol, chloramphenicol, gentamicin, kanamycin, spectinomycin, and tetracycline were all purchased from Sigma, St. Louis, Mo. Genistein was obtained from Fluka, Buchs, Germany.

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For experiments, overnight cultures grown at 26°C with appropriate antibiotics were diluted 1:20 in fresh

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TABLE 1. *Y. enterocolitica* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
WA-314	Serogroup O8; clinical isolate harboring virulence plasmid pYVO8	19
WA-C	Plasmidless derivative of strain WA-314	19
WA-C-*inv*	*inv* mutant of WA-C	This study
pYV-A-0	Insertional inactivation of *yadA* with kanamycin cassette	36
pYV-515	Tn*5* insertional inactivation of *lcrD*	This study
pYV-7146	Tn*7* insertional inactivation of *sycH* (formerly designated pYVO8::Tn*7*)	12
pLCR	Low-calcium response (*lcr*) region of pYVO8 cloned as 30-kb *Sal*I fragment in pSUP102	38
pSALI	*lcr yopE yopH* region of pYVO8 cloned as *Sal*I fragment in pSUP102	38
pB8-64	6-kb *Eco*RI fragment of pYVO8 encoding YopH cloned in pRK290	38
pB8-23	20-kb *Bam*HI fragment of pYVO8 encompassing *yopE*, *yopH*, and *yadA* cloned in pRK290	38

Luria-Bertani broth and incubated at 26 or 37°C for 2 h. The bacteria were then washed in phosphate-buffered saline and resuspended in a modified Krebs-Ringer buffer containing 132 mM NaCl, 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM glucose, pH 7.1. The antibiotics were supplemented at the following final concentrations: kanamycin, 50 μg/ml; tetracycline, 20 μg/ml; spectinomycin, 50 μg/ml; chloramphenicol, 20 μg/ml; gentamicin, 50 μg/ml.

Preparation of human PMNs. PMNs were isolated from peripheral blood obtained from healthy volunteers by a one-step separation method using Mono-Poly Resolving Medium (Flow Laboratories, Irvine, United Kingdom) as described previously (10). Cell viability was >98% as determined by dye exclusion. Isolated PMNs were resuspended in the modified Krebs-Ringer buffer described above.

Chemiluminescence (CL) assay. Activation of the neutrophil oxidative burst was measured as luminol-enhanced CL. The CL response was monitored with a microplate chemiluminometer (Hamamatsu Photonics, Herrsching, Germany) at 37°C in the presence of 5×10^{-5} M luminol (10). PMNs (10^5 per well) were incubated at 37°C for 30 min prior to addition of bacteria. Bacteria were mixed with PMNs at a multiplicity of infection (MOI) of 50:1 unless stated otherwise. The desired bacterial concentrations were adjusted by measuring the optical densities at 600 nm. To check the bacterial concentrations more accurately, serial dilutions from every sample were plated on Mueller-Hinton agar in each assay and CFU were counted after incubation at 26°C for 36 h. For characterization of the complement- and antibody-dependent CL responses, 5% pooled normal human serum (NHS) or 1% polyclonal heat-inactivated (56°C, 30 min) rabbit anti-YadA serum or rabbit anti WA-C serum (51) was added to each sample, respectively. Bacteria were preopsonized with 5% NHS or 1% anti-YadA serum at 37°C for 5 min prior to stimulation of PMNs. The absence of *Yersinia*-specific antibodies in NHS was monitored by enzyme-linked immunosorbent assay and immunoblotting with Yop antigen (13). The number of photon counts per 30 s was recorded for a total of 1.5 h. PMNs were then restimulated with opsonized zymosan (250 μg/ml) or phorbol myristate acetate (1 μg/ml) to investigate whether interaction of bacteria with PMNs had an effect on the subsequent ability of PMNs to generate a CL response. The appropriate quantity of the stimulating agent was added directly to the wells of the microplate. The secondary CL signal was measured for at least 40 min. Zymosan was opsonized by incubation with pooled, undiluted NHS for 20 min at 37°C and washed twice with phosphate-buffered saline. To discriminate between intra- and extracellular CL events, either superoxide dismutase (200 U/ml) and catalase (2,000 U/ml) or horseradish peroxidase (4 U/ml) and azide (1 mM) were added to the wells to quench extra- or intracellular oxygen metabolites, respectively (4, 8, 10, 30). In some experiments, PMNs were pretreated with the tyrosine kinase inhibitor genistein (15 μg/ml) prior to stimulation with opsonized zymosan. Each assay was performed in duplicate and repeated at least three times. Only representative and reproducible CL graphs are shown.

Adherence, phagocytosis, and killing assay. Adherence, phagocytosis, and killing experiments were performed in parallel with the CL assays. Bacteria were incubated with PMNs at 37°C in microplates at the same MOI as for the CL assay.

For adherence studies, PMNs were washed with PBS and centrifuged onto glass slides with a cytocentrifuge (Shandon, Pittsburgh, Pa.). Slides were air dried, fixed with methanol, and stained with Giemsa. The numbers of PMN-associated bacteria were determined by counting 100 PMNs from each experiment in a light microscope. Results are expressed as mean numbers of bacteria per PMN. The experiments were repeated at least three times. To discriminate between intra- and extracellularly located bacteria, a double-immunofluorescence technique described by Heesemann and Laufs (23) was used. Briefly, extracellularly located bacteria were marked first with anti-O8 rabbit serum and visualized with a tetramethyl rhodamine isocyanate-conjugated anti-antibody. After fixation and permeabilization of the cell membrane, intracellularly located bacteria were also treated with anti-O8 rabbit serum and stained subsequently with a fluorescein isothiocyanate-conjugated second antibody. This technique allows determination of the numbers of both cell-associated (fluorescing red and green) and ingested (fluorescing exclusively green) bacteria. For every strain investigated, at least three separate experiments were performed and 100 PMNs from each experiment were analyzed in a fluorescence microscope. The mean percentages of ingested versus total numbers of bacteria per PMN were determined. For evaluation of the ratio of killed *Y. enterocolitica* strains, PMNs were lysed with ice-cold distilled water containing 0.5% Tergitol after incubation with bacteria for 1.5 h at 37°C. Serial dilutions of this suspension were plated on Mueller-Hinton agar, and CFU were counted after 36 h of incubation at 26°C (number of survivors, *s*). Control samples, containing no PMNs, were treated in parallel under the same conditions (total number, *t*). The killing rate was quantified as the mean percentage (± the standard deviation) of killed bacteria ($t - s$) with respect to the total number of bacteria (t) from at least three independent experiments.

Determination of propidium iodide-stainable PMNs. To check membrane damage, PMNs were stained with propidium iodide after incubation with bacteria at 37°C for 1.5 h as described previously (43). Cells were analyzed for propidium iodide-mediated nuclear fluorescence by FACScan (Becton Dickinson, Darmstadt, Germany).

DNA methods and construction of mutant strains. DNA was isolated, digested with restriction endonucleases, and ligated by standard methods (42) in accordance with the recommendations of the manufacturers (Boehringer Mannheim Biochemicals, Pharmacia, and New England Biolabs). Transformations into *Escherichia coli* were performed by electroporation (Gene Pulse Apparatus; Bio-Rad Laboratories, Munich, Germany) in accordance with the manufacturer's instructions.

To construct a WA-C-*inv* mutant, we screened recombinant clones of our WA-C cosmid genomic library in vector pLAFR2 (36) for the presence of the *inv* gene by colony hybridization with an *inv* PCR probe. A 4-kb chromosomal *Nco*I-*Pst*I DNA fragment encompassing the *inv* gene encoding Inv was subcloned. Insertional inactivation of the *inv* gene was accomplished by introduction of a 1.2-kb *Bam*HI kanamycin resistance fragment derived from plasmid pUC-4K (Pharmacia) into the internal *Bgl*II site of the *inv* gene. The *inv*::*km* construct was transferred into suicide vector pGP704 (31). The resulting mutator plasmid was mobilized into strain WA-C by conjugation, and the chromosomal *inv* gene was replaced with the *inv*::*km* construct by double recombination. Allelic exchange was checked by Southern blot hybridization (data not shown).

Mutant strain WA-C(pYV-515), deficient in secretion of the Yops, was isolated by Tn*5* transposon mutagenesis. The Tn*5* insertion was localized within the *lcrD* gene of virulence plasmid pYVO8. LcrD is essential for Yop translocation through the bacterial membrane (35) but does not inhibit expression of YadA in WA-C(pYV-515), as could be demonstrated by indirect immunofluorescence with anti-YadA monoclonal antibodies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (results not shown; see also reference 37).

Insertional inactivation of the *sycH* gene of virulence plasmid pYVO8 was accomplished by Tn*7* transposon mutagenesis as described elsewhere (12). Resultant *sycH* mutant strain WA-C(pYV-7146) was affected in YopH secretion. The locations of transposon insertions were confirmed by restriction mapping,

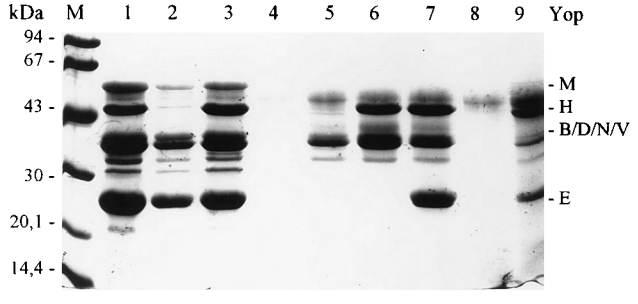

FIG. 1. Yop profile of *Y. enterocolitica* WA-314 (lane 1), *sycH* mutant WA-C(pVY-7146) (lane 2), WA-C(pYV-7146, pB8-64) (lane 3), *lcrD* mutant WA-C(pYV-515) (lane 4), WA-C(pLCR) (lane 5), WA-C(pLCR, pB8-64) (lane 6), WA-C(pLCR, pB8-23) (lane 7), WA-C (lane 8), and WA-C(pSALI) (lane 9) obtained with a Coomassie-stained sodium dodecyl sulfate–11% polyacrylamide gel. Lane M, prestained molecular size marker proteins.

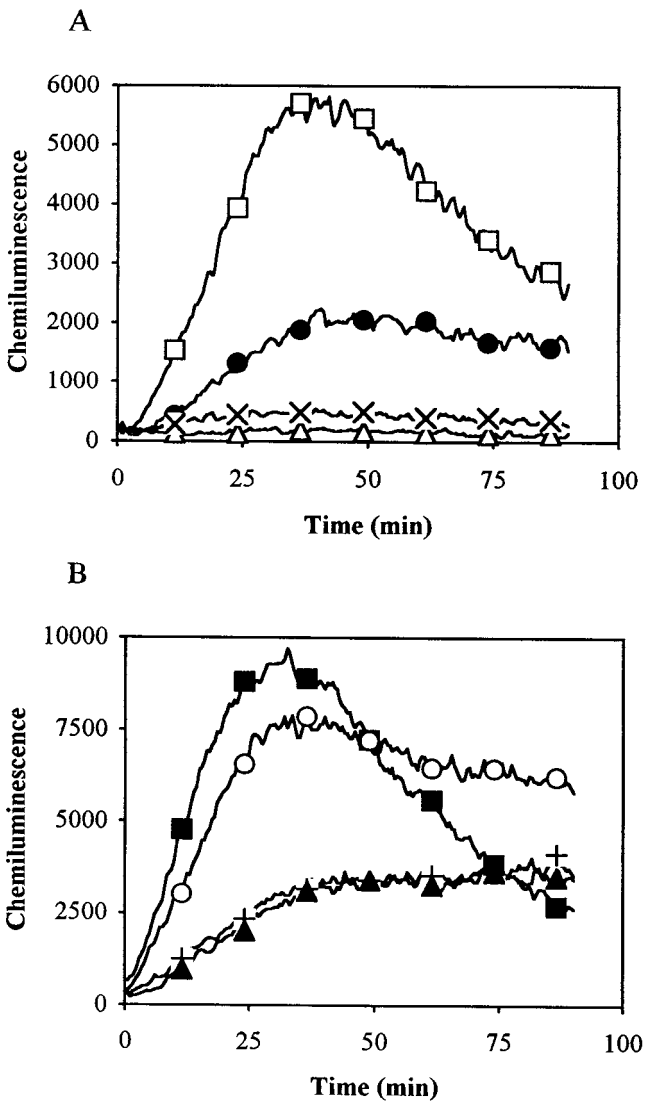

FIG. 2. Opsonin-independent CL responses of PMNs to *Y. enterocolitica*. *Yersinia* strains were grown at 27 and/or 37°C to determine the role of Inv (A: ●, WA-C at 37°C; △, WA-C-*inv* at 37°C; □, WA-C at 26°C; X, WA-C-*inv* at 26°C) and that of YadA [B: +, WA-C; ■, WA-314; ▲, *yadA* mutant WA-C(pYV-A-0), ○, *lcrD* mutant WA-C(pYV-515), all at 37°C]. PMNs were incubated with yersiniae at an MOI of 50:1 in the absence of serum opsonins.

PCR, and Southern blot hybridization (data not shown). Yop production and secretion were checked as described previously (12, 20; Fig. 1).

RESULTS

Opsonin-independent interaction with PMNs. We first analyzed the interaction between *Y. enterocolitica* and PMNs in the absence of serum. *Yersinia* strains were grown at 26 or 37°C and incubated with PMNs at a ratio of 50 bacteria per PMN. The CL response was monitored, and the numbers of cell-associated bacteria were determined.

Inv-mediated interaction. Expression of *inv* in vitro is maximally induced at room temperature (26 to 28°C) and decreases at 37°C (24, 33). Strain WA-C, not affected in Inv production, generated a CL response that was threefold higher when bacteria were grown at 26°C than when they were grown at 37°C (Fig. 2A). The *inv* mutant WA-C-*inv*, on the other hand, could not elicit a detectable CL response when grown at either temperature. The numbers of cell-associated bacteria, determined after 1 h of incubation, support the findings from the CL assay: strain WA-C grown at 26°C was most capable of interacting with PMNs (Table 2). Under these conditions, 57% of the cell-associated bacteria were intracellular, as demonstrated by double-immunofluorescence staining. These results indicate that Inv mediates adherence to PMNs and promotes phagocytosis and CL.

YadA-mediated interaction. At a growth temperature of 37°C, virulence plasmid-harboring strain WA-314 induced a marked and early CL response, in contrast to virulence plasmid-cured strain WA-C (Fig. 2B). Also, strain WA-C(pYV-515), impaired in Yop secretion but not affected in production of YadA (*lcrD* mutant), generated a rapid CL response. The *yadA* mutant WA-C(pYV-A-0), on the other hand, induced only a low and late CL signal similar to that of virulence plasmid-cured strain WA-C. The YadA-mediated CL response was associated with increased attachment of bacteria to PMNs (Table 2): YadA-bearing strains [WA-314 and WA-C(pYV-515)] were vigorously associated with PMNs after 15 and 45 min of incubation, respectively, in contrast to strains without YadA [WA-C and WA-C(pYV-A-0)]. These results indicate a significant contribution of YadA as an adherence factor for PMNs.

Interaction between *Y. enterocolitica* and PMNs after opsonization with NHS. In the next step, we analyzed the interaction of different *Y. enterocolitica* strains with PMNs in the presence of 5% NHS. Bacteria were grown at 37°C, opsonized, and incubated with PMNs at an MOI of 50:1. CL responses and rates of phagocytosis and killing were investigated. Interaction between PMNs and *Y. enterocolitica* increased in the presence of NHS: attachment to PMNs was enhanced, and *Y. enterocolitica* elicited markedly stronger CL signals. The majority (80 to 90%) of the initial bacteria were cell associated after, on average, 1 h of incubation with PMNs as demonstrated by Giemsa staining. In support of previous investigations (5, 10, 29), opsonized, plasmid-cured strain WA-C induced a strong CL response, in contrast to plasmid-bearing strain WA-314 (Fig. 3). The strong CL response generated by WA-C was composed of an early intracellular CL event and a subsequent extracellular CL event, while the weak CL response induced by WA-314 was only of intracellular origin (Fig. 4). These results are in good agreement with those obtained previously (10). The different CL signals induced by WA-314 and WA-C correlated with the rates of phagocytosis and killing for these strains (Table 3). Ninety-eight percent of PMN-associated strain WA-C bacteria were localized intracel-

TABLE 2. Quantitation of PMN-associated bacteria in the absence of serum opsonins[a]

Strain, temp (°C)	Mean no. of bacteria/PMN ± SD at incubation time of:		
	15 min	45 min	60 min
WA-C, 26	ND	ND	14.2 ± 3
WA-C-*inv*, 26	ND	ND	2.8 ± 1
WA-C, 37	1.6 ± 0.4	3.8 ± 1	4.5 ± 1
WA-C-*inv*, 37	ND	ND	1.2 ± 0.6
WA-314, 37	6.1 ± 2	11.1 ± 2	ND
WA-C(pYV-A-0), 37	1.7 ± 0.7	3.6 ± 1	ND
WA-C(pYV-515), 37	6.0 ± 2	10.9 ± 3	ND

[a] PMNs were incubated with bacteria at an MOI of 50:1 for 15, 45, or 60 min and subsequently stained with Giemsa. The number of bacteria associated with 100 PMNs was determined in each experiment. The experiments were repeated at least three times. ND, not done.

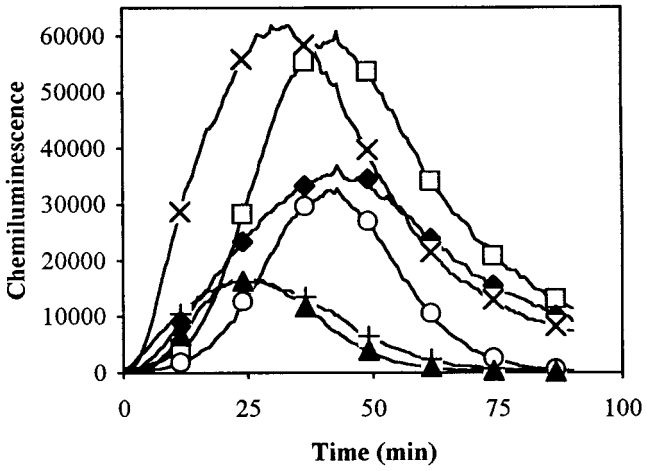

FIG. 3. CL response of PMNs to *Y. enterocolitica* in the presence of 5% NHS. Strains were grown at 37°C and incubated with PMNs at an MOI of 50:1. □, WA-C; +, WA-314; ○, *yadA* mutant WA-C(pYV-A-0); ◆, *sycH* mutant WA-C(pYV-7146); ▲, WA-C(pYV-7146, pB8-64); ×, *lcrD* mutant WA-C(pYV-515).

lularly, in contrast to virulence plasmid-harboring strain WA-314 bacteria, which were predominantly resistant to phagocytosis (Fig. 5): only 12% of bacteria were ingested, and the remaining bacteria were located extracellularly at the granulocyte surface. After 1.5 h of incubation, strain WA-C was nearly completely (94%) killed, unlike strain WA-314 bacteria, of which 96% survived. We used diverse mutants of strain WA-314 affected in the production of different plasmid-encoded virulence factors for more detailed analysis of the impact of the *Yersinia* virulence plasmid on interaction with PMNs. YadA is supposed to play a primary role in resistance to phagocytosis by PMNs in the presence of complement (5). In line with these findings, the *yadA* mutant WA-C(pYV-A-0) induced a stronger CL response than wild-type strain WA-314 (Fig. 3). On the other hand, the CL signal generated by the *lcrD* mutant WA-C(pYV-515), which produces YadA but is deficient in Yop secretion, was even stronger than that of strain WA-C(pYV-A-0). Interestingly, the development of the CL signal of WA-C(pYV-515) was more rapid, comparable to that of wild-type strain WA-314 (maximum after about 25 to 30 min), whereas the CL signals of the *yadA* mutant and strain WA-C did not culminate before 45 min. Thus, under opsonizing conditions, *yadA*-expressing strains generated a more rapid CL response than did strains lacking YadA, suggesting that YadA is involved in promotion of adherence to PMNs. Complementation of strain WA-C(pYV-A-0) with *yadA* in *trans* resulted in a CL response similar to that of wild-type strain WA-314 (data not shown).

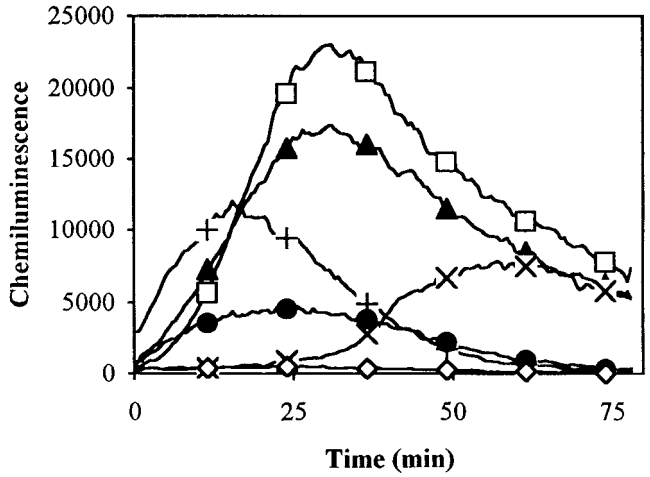

FIG. 4. Intra- and extracellular CL responses of PMNs to *Y. enterocolitica* WA-314 and WA-C. Extra- and intracellular oxygen metabolites were quenched with superoxide dismutase and catalase (intracellular CL responses: ▲, WA-C; ●, WA-314) or horseradish peroxidase and azide (extracellular CL responses: ×, WA-C; ◇, WA-314). Unquenched controls: □, WA-C; +, WA-314.

Emphasizing the importance of YadA in the interaction with PMNs, the *yadA* mutant WA-C(pYV-A-0) was less resistant to phagocytosis and killing than was wild-type strain WA-314: 44% of strain WA-C(pYV-A-0) bacteria were ingested, and 47% were killed (Table 3). According to the strong CL response, strain WA-C(pYV-515) was efficiently phagocytosed (90%) and killed (78%). Supporting previous findings (50), these results demonstrate that the presence of YadA alone has no effect on inhibition of phagocytosis and killing. Other plasmid-encoded virulence proteins must be involved in interaction with PMNs.

Strain WA-C(pYV-7146), affected in secretion of YopH (*sycH* mutant), induced a rapid CL response, probably due to expression of *yadA* (Fig. 3), but the CL signal was markedly stronger than that of wild-type strain WA-314. It was associated with high rates of phagocytosis (65%) and killing (71%) of *Y. enterocolitica* (Table 3). For complementation, plasmid pB8-64, carrying the *yopH* and *sycH* genes, was mobilized into strain WA-C(pYV-7146). Strain WA-C(pYV-7146, pB8-64) induced only a weak CL signal (Fig. 3) and was predominantly resistant to phagocytosis and killing (Table 3). These results demonstrate that YopH tyrosine phosphatase activity is highly involved in preventing phagocytosis and killing of yersiniae by PMNs in the presence of NHS.

Inhibition of the secondary zymosan-induced CL response by wild-type and mutated *Y. enterocolitica* strains. To investigate whether interaction of *Y. enterocolitica* with PMNs has an effect on the subsequent ability of PMNs to generate an oxidative burst, PMNs were restimulated with opsonized zymosan after incubation with bacteria and CL was monitored (secondary CL response). *Y. enterocolitica* strains were grown at 37°C and incubated with PMNs at 37°C for 1.5 h in the presence of 5% NHS (see above) before opsonized zymosan was added. The secondary zymosan-induced CL response was almost completely inhibited when PMNs were preexposed to virulence plasmid-harboring strain WA-314 (Fig. 6A). In contrast, PMNs incubated with strain WA-C responded with a rapid and strong CL signal after secondary stimulation with opsonized zymosan. Nevertheless, strain WA-314 did not block binding of opsonized zymosan to PMNs, which was proven by Giemsa staining (data not shown). Secondary CL response inhibition by strain WA-314 depended on the time of bacterial interaction and on the number of cell-associated bacteria. At an MOI of 50:1, the secondary CL response was reduced by more than 50% after 30 min of incubation (Fig. 7A). It was completely inhibited after 1.5 h of bacterial interaction. Strain WA-C caused only modest secondary CL response inhibition within this period of time. Furthermore, we observed 50% secondary CL signal reduction by strain WA-314 at an MOI of 5:1, whereas 20 to 30 bacteria reduced the CL response completely (Fig. 7B). In every sample, 80 to 90% of the initial bacteria were cell associated after, on average, 1 h of incubation with PMNs as proven by Giemsa staining. Inhibition of the secondary CL response by strain WA-314 was also observed in the absence of NHS (data not shown), but inhibition occurred to a lesser extent than with opsonized bacteria, probably because of

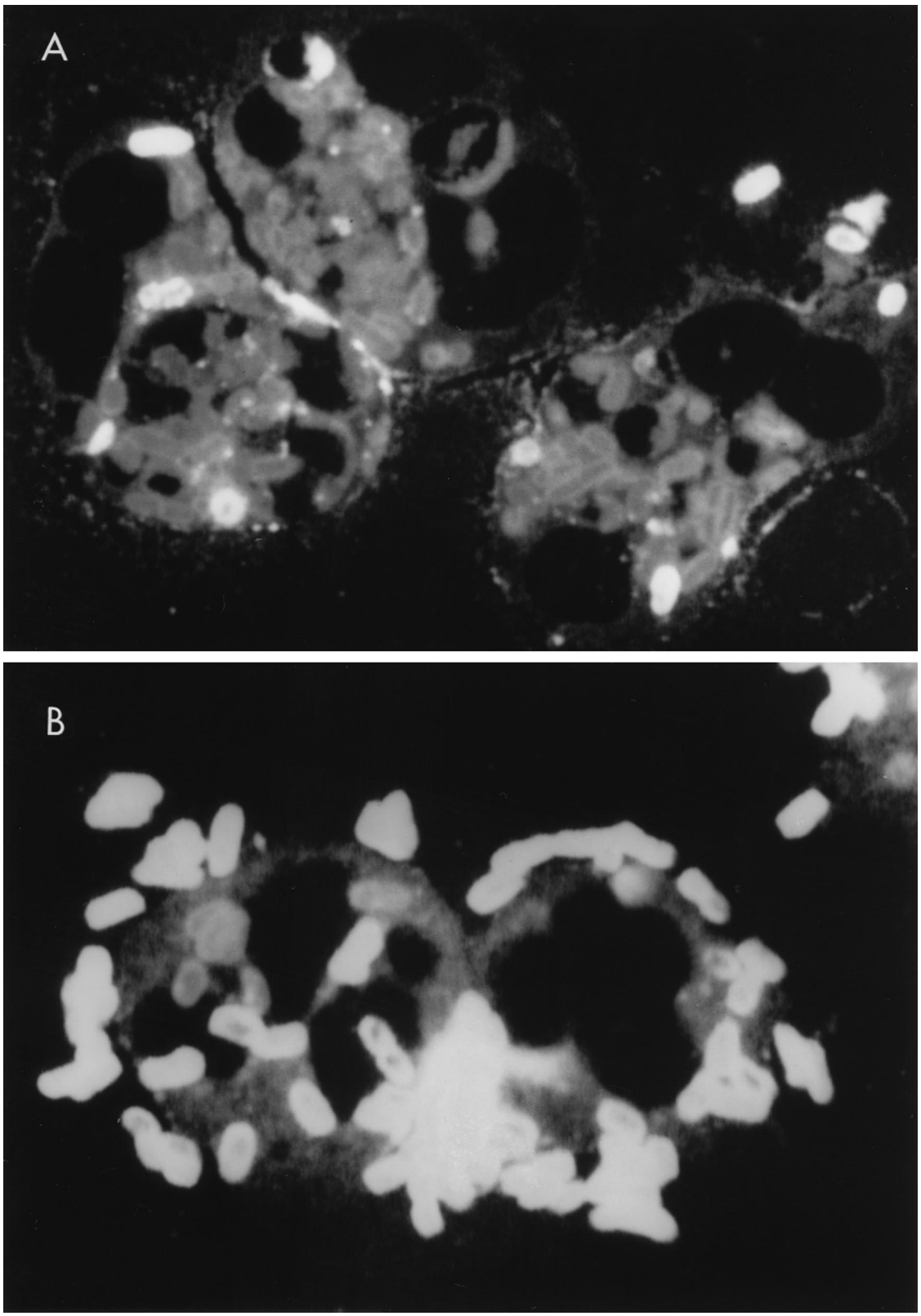

FIG. 5. Location of *Y. enterocolitica* WA-C (A) and WA-314 (B) on PMNs after 1 h of interaction in the presence of 5% NHS. Cells were stained by a double-immunofluorescence technique. Intracellular bacteria are dark, and extracellular bacteria are bright. Cells were analyzed by confocal laser scanning microscopy (TCS 4D; Leica, Heidelberg, Germany).

TABLE 3. Phagocytosis and killing of *Y. enterocolitica* strains by PMNs

Strain	Mean % phagocytosis[a] ± SD	Mean % killing[b] ± SD
WA-C	98 ± 1	94 ± 9
WA-314	12 ± 3	4 ± 14
WA-C(pYV-A-0)	44 ± 7	47 ± 19
WA-C(pYV-515)	90 ± 5	78 ± 12
WA-C(pYV-7146)	65 ± 8	71 ± 17
WA-C(pYV-7146, pB8-64)	11 ± 3	3 ± 5
WA-C(pLCR)	89 ± 5	70 ± 18
WA-C(pLCR, pB8-64)	50 ± 8	42 ± 18
WA-C(pSALI)	39 ± 5	36 ± 13
WA-C(pLCR, pB8-23)	5 ± 2	3 ± 12

[a] PMNs were incubated with different strains in the presence of 5% NHS at 37°C (MOI, 50:1). After 1 h of incubation, PMN-associated bacteria were stained by using a double-immunofluorescence technique to discriminate between intra- and extracellular localizations. Mean percentages of ingested bacteria with respect to the total number of bacteria per PMN were determined by counting 100 PMNs from each experiment. The experiments were repeated at least three times.

[b] For killing assays, bacteria were incubated with or without PMNs (control sample) in parallel. After 1.5 h, PMNs were lysed and numbers of viable bacteria were determined by plating. The killing rate was quantified as the mean percentage of killed bacteria with respect to the total number of bacteria, derived from the control sample, from at least three independent experiments.

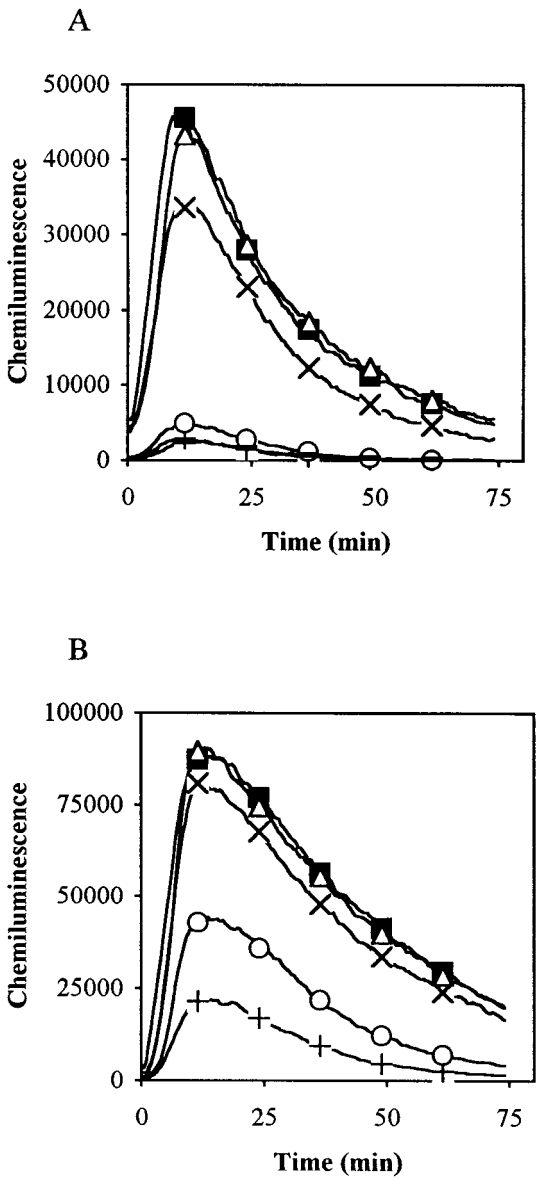

FIG. 6. Impact of *Y. enterocolitica* strains on inhibition of the secondary zymosan-induced CL response. PMNs were preexposed to yersiniae at an MOI of 50:1 (A) or 10:1 (B) for 1.5 h at 37°C in the presence of 5% NHS. Thereafter, PMNs were treated with opsonized zymosan and the CL responses were monitored. ■, WA-C; +, WA-314; ○, *yadA* mutant WA-C(pYV-A-0); ×, *sycH* mutant WA-C(pYV-7146); —, WA-C(pYV-7146, pB8-64); △, *lcrD* mutant WA-C(pYV-515).

weaker and delayed interaction with PMNs. Interestingly, CL response inhibition depended on the nature of the secondary stimulus: PMNs incubated with strain WA-314 could not be restimulated with either opsonized zymosan or opsonized bacteria (maximum CL, ≈ 800 photon counts), but weak restimulation was possible by treatment with the protein kinase C activator phorbol myristate acetate (maximum CL, ≈ 3,000 photon counts) (data not shown).

To test whether CL inhibition is reversible, extracellular bacteria were killed with gentamicin after incubation with PMNs for 1.5 h. PMNs were then treated with opsonized zymosan 1, 2, 4, and 6 h later, respectively. Zymosan treatment did not result in a significant CL response when PMNs were preexposed to strain WA-314, demonstrating prolonged inhibition of the oxidative burst. We did not investigate the ability of PMNs to respond with a CL signal after a time longer than 6 h because of the reduced viability of PMNs when they are cultured in vitro for a longer period. PMNs incubated with strain WA-C for 1.5 h revealed a higher proportion of propidium iodide-positive cells (12.8%) than did PMNs incubated with strain WA-314 (3.8%) or noninfected cells (4.6%). Thus, strain WA-314 obviously does not cause significant membrane damage of PMNs.

The *yadA* mutant WA-C(pYV-A-0) demonstrated markedly lower CL inhibition than strain WA-314 at an MOI of 10 bacteria per PMN (Fig. 6B) but not at an MOI of 50 bacteria per PMN (Fig. 6A). About 40 to 60 strain WA-C(pYV-A-0) bacteria were necessary for complete CL inhibition, twice as much as strain WA-314 bacteria (data not shown). These results indicate that YadA also plays a role in CL inhibition. However, the *lcrD* mutant WA-C(pYV-515), producing YadA but deficient in Yop secretion, failed to inhibit the secondary zymosan-induced CL response, and PMNs generated the typical CL signal (Fig. 6A and B). Strain WA-C(pYV-7146), which is impaired only in YopH secretion, was also affected in the ability to reduce the secondary oxidative burst: PMNs incubated with WA-C(pYV-7146) could be restimulated to almost the same extent as PMNs incubated with WA-C or WA-C(pYV-515) (Fig. 6A and B). In contrast, strain WA-C(pYV-7146, pB8-64) complemented with *yopH* and *sycH* was strongly able to inhibit the secondary CL response (Fig. 6A). Thus, YopH obviously plays a major role in CL inhibition in the presence of NHS. To determine the role of tyrosine phosphorylation in the generation of a CL signal, PMNs were incubated with the tyrosine kinase inhibitor genistein (dissolved in dimethyl sulfoxide) for 1.5 h or 5 min and subsequently treated with opsonized zymosan. No CL response was obtained in any case (data not shown). Incubation with the corresponding amount of dimethyl sulfoxide alone had no effect on CL generation. These results support previous findings that tyrosine phosphorylation is essential in the signalling pathways involved in NADPH oxidase activation (16). YopH seems to interfere

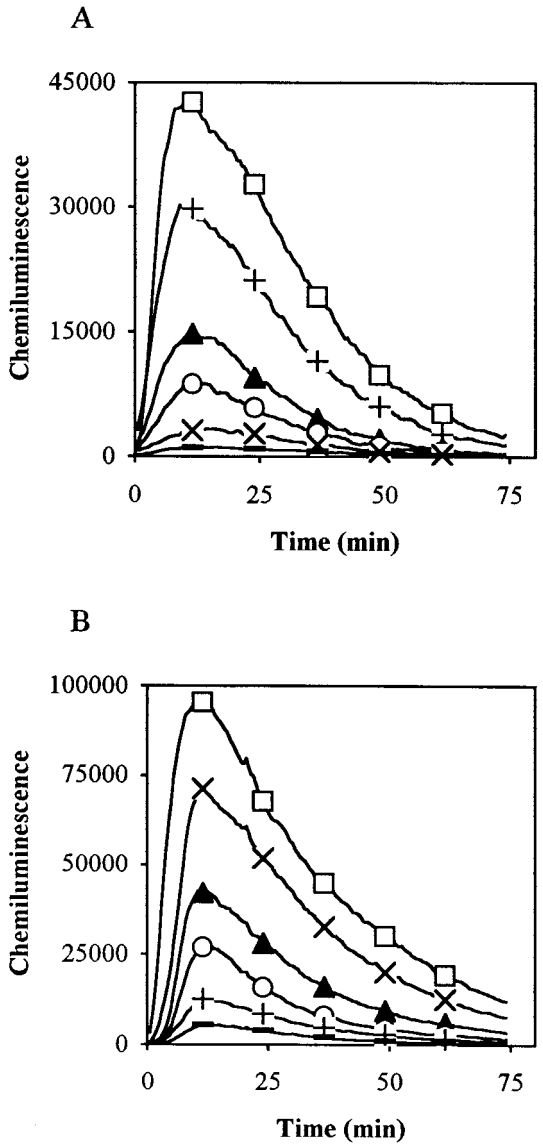

FIG. 7. Time- and bacterial concentration-dependent impact of strain WA-314 on the secondary zymosan-induced CL response. PMNs were preexposed to strain WA-314 for various periods of time at an MOI of 50:1 (A) (times: □, 1 min; +, 15 min; ▲, 30 min; ○, 45 min; ×, 60 min; —, 90 min) and at various MOIs for a period of 1.5 h (B) (yersinia-PMN ratios: □, 1:1; ×, 2:1; ▲, 5:1; ○, 10:1; +, 20:1; —, 30:1). Thereafter, PMNs were treated with opsonized zymosan and the CL responses were monitored.

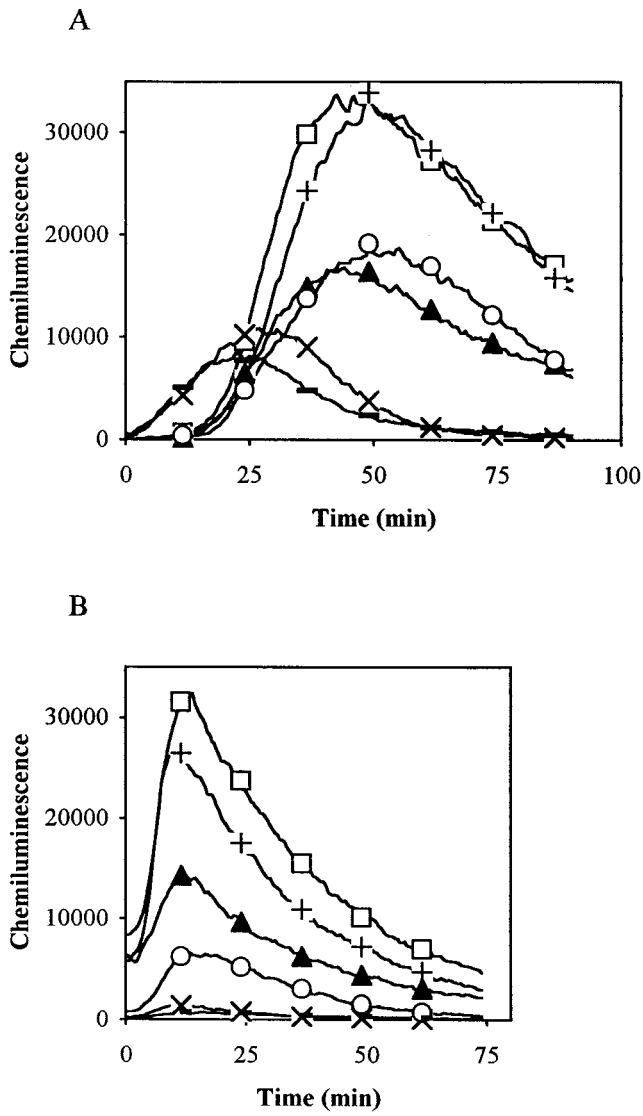

FIG. 8. Impact of definite plasmid-encoded virulence factors on the primary *Yersinia* (A)- and secondary zymosan (B)-induced CL responses. Strains were grown at 37°C and incubated with PMNs for 1.5 h (MOI, 50:1). PMNs were subsequently treated with opsonized zymosan. Both CL signals were monitored. □, WA-C; ×, WA-314; +, WA-C(pLCR); ▲, WA-C(pLCR, pB8-64); ○, WA-C (pSALI); —, WA-C(pLCR, pB8-23).

with this pathway and thus reduces the ability of PMNs to generate an oxidative burst.

Roles of individual plasmid-encoded virulence factors in the interaction between *Y. enterocolitica* and PMNs. To determine the roles of the Yops and YadA in the interaction between *Y. enterocolitica* and PMNs in the presence of NHS more precisely, we constructed *Y. enterocolitica* strains which produce individual plasmid-encoded virulence proteins (38). Plasmid pLCR encodes the low-calcium response (*lcr*) region of pYVO8, which is essential for regulation of Yop production and polarized translocation. Strain WA-C(pLCR), secreting only YopD, YopB, YopN, and V-antigen, induced a strong CL signal, comparable to that of strain WA-C (Fig. 8A). The secondary CL response was inhibited only a little (Fig. 8B). To analyze the impact of YopH on interaction with PMNs, we additionally introduced plasmid pB8-64, encoding *yopH* and *sycH*, into strain WA-C(pLCR). The resulting strain, WA-C(pLCR, pB8-64), generated a reduced primary CL signal (Fig. 8A) and suppressed the secondary CL response (Fig. 8B). Further reduction of the secondary CL signal was demonstrated for strain WA-C(pSALI), which is capable of secreting YopH and YopE (Fig. 8B; plasmid pSALI carries the *lcr* region plus *yopH-sycH* and *yopE-sycE*). Thus, in addition to YopH, YopE also participates in reduction of the oxidative burst of PMNs. Plasmid pB8-23, encompassing *yopH-sycH*, *yopE-sycE*, and *yadA*, was mobilized in strain WA-C(pLCR) to investigate the influence of YopH, YopE, and YadA on PMNs in concert. The resulting strain, WA-C(pLCR, pB8-23), induced a weak but rapid primary CL signal, similar to that of wild-type strain WA-314 (Fig. 8A). The secondary CL re-

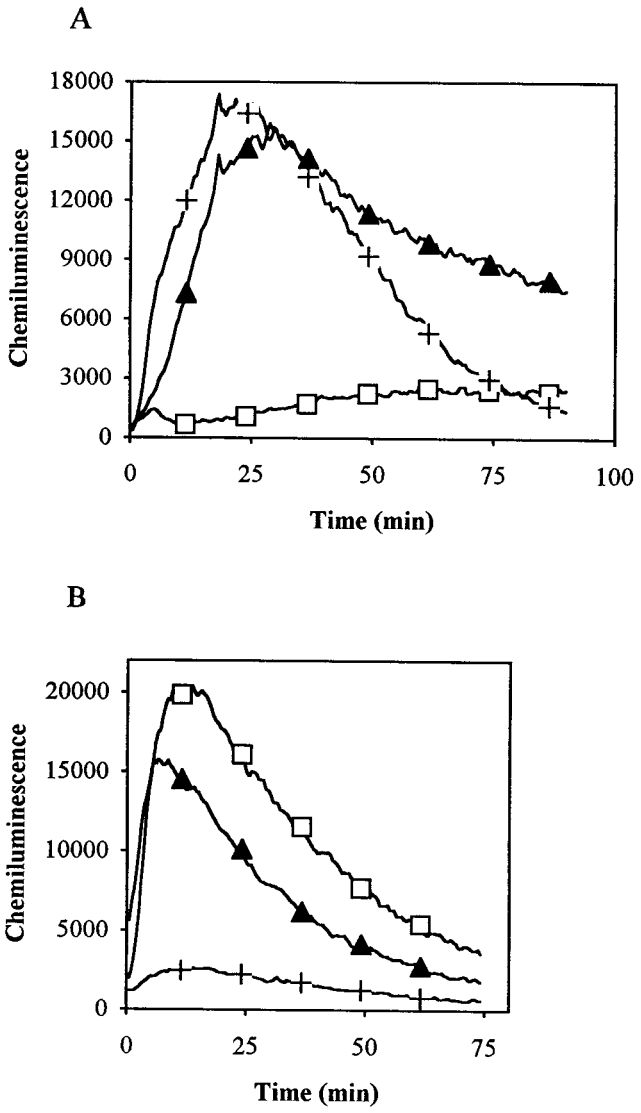

FIG. 9. Effect of antibody opsonization on the primary *Yersinia* (A)- and secondary zymosan (B)-induced CL responses. Bacteria were grown at 37°C and incubated with PMNs in the presence of 1% heat-inactivated polyclonal rabbit anti-*Y. enterocolitica* YadA serum (MOI, 50:1). After 1.5 h, PMNs were treated with opsonized zymosan. Both CL signals were monitored. □, WA-C; +, WA-314; ▲, *lcrD* mutant WA-C(pYV-515).

sponse was completely inhibited (Fig. 8B). These results support our finding that YadA enhances the interaction with PMNs, and this seems to be essential for effective CL inhibition. Moreover, YopH, YopE, and YadA also had an impact on the phagocytosis and killing of *Y. enterocolitica* by PMNs, as shown in Table 3. Strain WA-C(pLCR, pB8-23) was ingested and killed even less effectively than wild-type strain WA-314. We conclude from these results that YopH and YadA, and also YopE, act in concert to inhibit the oxidative burst of PMNs. Furthermore, YopH, YopE, and YadA are of major importance in preventing phagocytosis and killing of *Y. enterocolitica* by PMNs.

Inhibition of the secondary zymosan-induced CL response after opsonization with antibodies. We performed experiments with antibody-opsonized bacteria to elucidate whether virulent *Y. enterocolitica* also causes CL inhibition in the presence of antibodies. *Y. enterocolitica* was opsonized with 1% heat-inactivated polyclonal rabbit anti-YadA serum (51) and incubated with PMNs at a ratio of 50 bacteria per PMN. YadA-bearing strains WA-314 and WA-C(pYV-515) induced a strong CL response, in contrast to YadA-lacking strain WA-C (Fig. 9A). Antibody binding to YadA-bearing strains was confirmed by immunofluorescence staining with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibodies (data not shown). The secondary CL response of PMNs incubated with strain WA-314 was almost completely inhibited, in contrast to that of PMNs incubated with *lcrD* mutant WA-C(pYV-515) (Fig. 9B). Thus, secondary CL response inhibition by the Yop proteins seems to be independent of the mode of bacterial interaction (mediated by YadA, complement, or antibodies). Similar results have been obtained with serotype O:8-specific serum (anti-WA-C) lacking YadA-specific antibodies (data not shown; 51).

DISCUSSION

In the present study, we analyzed the complex interaction of *Y. enterocolitica* with PMNs (CL, phagocytosis, and killing). Serum opsonin-dependent experiments were performed under opsonizing conditions with 5% NHS as described by Lian and Pai (29). They were the first to demonstrate the importance of the *Y. enterocolitica* virulence plasmid in evasion of antibacterial functions of PMNs. Our experiments confirmed their findings that virulence plasmid-bearing, in contrast to plasmid-cured, *Y. enterocolitica* strains elicit only a weak CL response and are highly resistant to neutrophil phagocytosis (28, 29). Moreover, *Y. enterocolitica* strains harboring the virulence plasmid evaded killing by PMNs and were located predominantly extracellularly, at the granulocyte surface. Interaction of virulent *Y. enterocolitica* with PMNs reduced the ability of PMNs to generate an oxidative burst upon stimulation with opsonized zymosan in a time- and bacterial concentration-dependent manner. Inhibition of the oxidative burst lasted for at least 6 h, but incubation with virulent *Y. enterocolitica* did not cause membrane damage of PMNs, which was determined by propidium iodide staining. PMNs exposed to virulence plasmid-cured *Y. enterocolitica* revealed an even higher proportion of propidium iodide-positive cells than did PMNs incubated with the plasmid-bearing strain. The better propidium iodide staining pattern of PMNs infected with plasmid-cured *Y. enterocolitica* seems to correlate with the extracellular CL event observed for these granulocytes and may be a result of the high phagocytic activity associated with cell damage and extracellular release of oxygen metabolites.

However, it is still uncertain which *Y. enterocolitica* virulence factors are involved in interaction with PMNs. Lian et al. (28, 29) demonstrated that resistance to phagocytosis and lack of the oxidative burst are mediated by outer membrane fragments of virulent *Y. enterocolitica*. Tertti et al. (49) found a correlation between the reduced CL response and diminished fixation of complement component C3b on the bacterial surface. There is evidence that the YadA protein prevents opsonization of *Y. enterocolitica* with complement by binding complement-inhibiting factor H, which degrades surface-bound C3b into C3bi (6, 34). China et al. (5) hypothesized that this pathway is responsible for resistance of *Y. enterocolitica* to complement-mediated phagocytosis by PMNs. They showed that *yadA* mutants are unable to elicit a reduced CL response in the presence of 20% NHS, in contrast to strains affected in the secretion of one or more Yops. Furthermore, phagocytosis and killing experiments with bacteria opsonized with Ca^{2+}-chelated NHS (inhibition of the classical complement pathway) revealed that

strains lacking YadA are highly susceptible to ingestion and killing. However, they did not investigate phagocytosis and killing of strains affected in Yop production. Visser et al. (50) found that not production of YadA but secretion of Yop proteins seems to inhibit phagocytic uptake and killing of *Y. enterocolitica*. In those experiments, bacteria were opsonized with rabbit anti-*Y. enterocolitica* antibodies and complement. They hypothesized that the differences from the results of China et al. (5) are based on the different opsonizing conditions. In contrast to macrophages, opsonization of the target seems to be prerequisite for strong interaction with PMNs (5, 29, 49). In our studies also, addition of 5% NHS enhanced interaction between PMNs and *Y. enterocolitica* to a great extent. We demonstrated that YopH, which counteracts phagocytosis of *Y. pseudotuberculosis* by macrophages (39), also plays an important role in interaction between *Y. enterocolitica* and PMNs. In contrast to wild-type *Y. enterocolitica*, a mutant strain affected in YopH secretion was highly susceptible to phagocytosis and killing by PMNs under opsonizing conditions. Moreover, this strain almost completely failed to inhibit the secondary zymosan-induced CL response, indicating that YopH phosphotyrosine phosphatase activity is also involved in oxidative burst inhibition. There is evidence that tyrosine phosphorylation is an important step in activation of neutrophil NADPH oxidase (16). This finding was confirmed by the observation that addition of the tyrosine kinase inhibitor genistein completely blocked generation of the zymosan-induced CL response. Thus, YopH interferes with the signalling pathways involved in NADPH oxidase activation. However, protein kinase C-dependent activation of the NADPH oxidase with phorbol myristate acetate was still possible to some extent, indicating that the *Yersinia* virulence factors at least partially block NADPH oxidase itself and probably do not act efficiently downstream of protein kinase C activation.

We further showed that the YadA protein is also essential for interaction between *Y. enterocolitica* and PMNs. In the presence of NHS, YadA contributed to inhibition of the oxidative burst of PMNs and to prevention of the phagocytosis and killing of *Y. enterocolitica*. YadA-bearing strains elicited a more rapid CL response than did strains lacking YadA, suggesting that YadA enhances interaction between *Y. enterocolitica* and PMNs. In the absence of serum opsonins, YadA directly mediated adherence to granulocytes, which was associated with a weak but clearly detectable CL response. Tertti et al. (49) observed that nonopsonized *Y. enterocolitica* strains harboring the virulence plasmid induced a higher CL response than did plasmid-free strains, and they hypothesized that plasmid-bearing strains are more adhesive to PMNs. However, further investigation is needed to determine which host cell structures are involved in YadA-mediated adherence to granulocytes. On the other hand, it is not known if YadA-mediated interaction with PMNs in the presence of NHS is a result of direct adherence to granulocytes or if interaction is affected by altered opsonizing conditions conferred by YadA itself. Possibly, large numbers of C3bi molecules obtained from cleavage of C3b by YadA-bound factor H favors binding to PMNs via CR3 receptors, which may activate altered signalling pathways.

Besides YadA, the Inv protein was also able to mediate interaction with PMNs in the absence of serum opsonins. The role of Inv in interaction with professional phagocytes has been recently described by Fällman et al. (11). In those experiments, Inv of *Y. pseudotuberculosis* induced ingestion of nonopsonized bacteria by the macrophage-like cell line J774. We found that Inv also confers adherence to PMNs and subsequently promotes phagocytosis, which coincides with a low but marked CL response. However, after addition of NHS, we could not demonstrate a contribution of Inv to the opsonin-dependent interaction with PMNs (data not shown), indicating that Inv plays no significant role under opsonizing conditions.

The results we obtained in experiments with *Y. enterocolitica* strains producing individual plasmid-encoded virulence factors confirmed our finding that both YopH and YadA are essential in mediation of CL inhibition and resistance to phagocytosis and killing. Moreover, we found that in addition to YopH and YadA, YopE is also involved in interaction with PMNs. The *Y. enterocolitica* strain secreting YopE in addition to YopH was more able to reduce the oxidative burst and to prevent phagocytosis and killing than was the strain unable to express *yopE*. An antiphagocytic activity of YopE upon macrophages has been described previously (40). The pathway by which YopE interferes with neutrophil functions is not known, but it is conceivable that disruption of actin microfilaments by YopE affects phagocytosis of bacteria and assembly of the NADPH oxidase, resulting in suppression of the respiratory burst. Also, Green et al. (14) suppose that a plasmid-encoded virulence protein(s) other than YopH is involved in inhibition of the macrophage respiratory burst. We conclude from our results that YopH, YopE, and YadA act in concert to resist antibacterial activities of PMNs under opsonizing conditions. We hypothesize that YadA is, first of all, involved in the mode of adherence to PMNs and inhibition of the bactericidal functions is caused predominantly by YopH and, to a certain extent, also by YopE.

Fällman et al. (11) found that *Y. pseudotuberculosis* resisted phagocytosis by J774 cells not only in the absence of serum opsonins but also when bacteria were opsonized with immunoglobulin G. Yop-dependent inhibition of the neutrophil respiratory burst by antibody-opsonized *Y. enterocolitica* was also demonstrated in our experiments, indicating that yersiniae are able to interfere with antibacterial functions of professional phagocytes even after binding via Fc receptors. This ability probably contributes to evasion of the specific immune defense of the host and favors extracellular location of yersiniae, even in phagocyte- and antibody-rich lymphoid tissue. In summary, we have evidence that YopH, in cooperation with YopE and YadA, can be considered a microbial tranquillizer for neutrophils.

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