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Inhibition of the Fc Receptor-Mediated Oxidative Burst in Macrophages by the *Yersinia pseudotuberculosis* Tyrosine Phosphatase

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Suppression of host-cell-mediated immunity is a hallmark feature of *Yersinia pseudotuberculosis* infection. To better understand this process, the interaction of *Y. pseudotuberculosis* with macrophages and the effect of the virulence plasmid-encoded *Yersinia* tyrosine phosphatase (YopH) on the oxidative burst was analyzed in a chemiluminescence assay. An oxidative burst was generated upon infection of macrophages with a plasmid-cured strain of *Y. pseudotuberculosis* opsonized with immunoglobulin G antibody. Infection with plasmid-containing *Y. pseudotuberculosis* inhibited the oxidative burst triggered by secondary infection with opsonized bacteria. The tyrosine phosphatase activity of YopH was necessary for this inhibition. These results indicate that YopH inhibits Fc receptor-mediated signal transduction in macrophages in a global fashion. In addition, bacterial protein synthesis was not required for macrophage inhibition, suggesting that YopH export and translocation are controlled at the posttranslational level.

A number of pathogenic bacteria are able to evade the bactericidal functions of phagocytes. Among these are the causative agents of bubonic plaque (*Yersinia pestis*) (9), tuberculosis (*Mycobacterium tuberculosis*) (1), and Legionnaires' disease (*Legionella pneumophila*) (29). Although the precise mechanisms of bacterial evasion are not fully understood, several common themes are emerging. Several bacteria appear to use integrin receptors to attach to and enter into macrophages (32, 38, 45). Unlike pathways mediated by receptors for immunoglobulin G (IgG) (Fc receptors), uptake via integrins may allow bacteria to evade the oxidative burst and/or enter into an intracellular compartment devoid of lysosomal enzymes (1, 13, 29, 32). Events that occur subsequently to receptor binding may also determine the fate of the bacterium. Certain bacteria export highly specialized proteins (23, 27) that act to counteract uptake by harmful phagocytic pathways (34), allow escape from phagocytic vacuoles (28), or induce programmed cell death (51).

Yersinia pseudotuberculosis is an enteric pathogen responsible for acute enteritis and mesenteric lymphadenitis in humans, other mammals, and birds (8). Yersiniae in contaminated food or drink initiate infection when they reach the ileum and penetrate intestinal lymphoid follicles (Peyer's patches) (16, 24). Invasin, an outer membrane protein that recognizes multiple β1 integrin receptors (32), is necessary for efficient penetration of Peyer's patches (39, 46, 47). Colonization of Peyer's patches and other lymphatic tissues requires a number of additional factors encoded by a 70-kb virulence plasmid (18, 41). Among these are a set of plasmid-encoded *Yersinia* outer membrane proteins (Yops) (6, 48) that are synthesized during growth at 37°C and exported upon contact with mammalian cells (15, 44). One of these is a 51-kDa protein with tyrosine-specific phosphatase activity, known as YopH (22). YopH dephosphorylates multiple targets in eukaryotic cells (5) and inhibits bacterial uptake by cultured macrophages (43) and epithelial cells (2). These findings suggest that YopH is active against host cell signalling pathways involved in bacterial uptake and killing (5, 43). We tested this model by measuring the effect of YopH on the signal transduction pathway(s) that activates the oxidative burst in macrophages.

Phagocytic cells emit light when ingesting opsonized bacteria as a result of the production of oxygen radicals (12). Oxidation of the cyclic hydrazide 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol) by reactive oxygen species can be used to enhance the level of measurable phagocyte chemiluminescence (12). Luminol-dependent chemiluminescence thus provides a convenient assay for phagocytic function as well as opsonization efficiency (12). Luminol-dependent chemiluminescence assays have been used previously to study the resistance of yersiniae to opsonization by complement (10, 35, 50). To use luminol-dependent chemiluminescence as an assay in our study of *Y. pseudotuberculosis*-macrophage interactions, we first needed to define the type of interaction(s) that triggers the oxidative burst. For this purpose, macrophages were challenged with the plasmid-cured *Y. pseudotuberculosis* strain YP137 (42), which expresses invasin and was either opsonized or unopsonized with IgG antibody. To prepare bacteria for assay, an overnight culture of YP137 grown at 26°C in Luria-Bertani (LB) broth was subcultured into LB to an optical density at 600 nm (OD_{600}) of 0.1 and was grown at 26°C with shaking to an OD_{600} of 0.5. These growth conditions have been shown to promote invasin expression in *Y. pseudotuberculosis* (31). Bacteria were pelleted by centrifugation and resuspended in Hanks balanced salt solution (HBSS; GIBCO) to an OD_{600} of 1.0 (10^9 CFU/ml). YP137 was coated with IgG by mixing 0.05 ml of bacteria with 0.02 ml of undiluted hybridoma supernatant containing the neutralizing monoclonal antibody 3A2 (IgG2a), which binds to invasin on the surface of *Y. pseudotuberculosis* (33). Macrophages were prepared for assaying by incubating confluent monolayers of J774A.1 cells in phosphate-buffered saline with 0.5 mM EDTA for 30 min at 37°C, and detached cells were pelleted by centrifugation and resuspended to a density of 10^6 cells per ml in HBSS containing 5×10^{-5} M Luminol (Sodium salt; Sigma). Glass culture tubes (12 by 75 mm) containing 1 ml of macrophage suspen-

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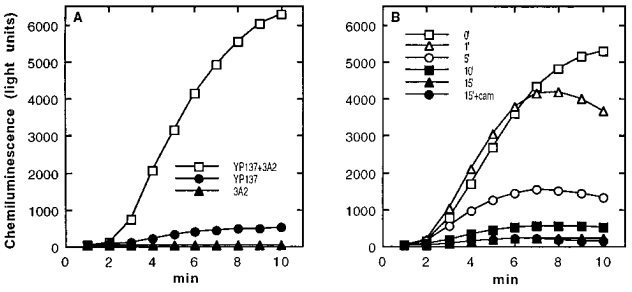

FIG. 1. Measurement of chemiluminescence from macrophages infected with *Y. pseudotuberculosis* strains. (A) Effect of antibody opsonization on chemiluminescence. At time zero, macrophages were mixed with YP137, YP137 opsonized with anti-invasin monoclonal antibody 3A2, or 3A2 alone, and chemiluminescence (light units) was measured for 2-s intervals over a time period of 10 min. The MOI was 50:1. (B) Time-dependent effect of YP126 infection on Fc receptor-mediated chemiluminescence. Macrophages were preincubated with YP126 (MOI = 50:1) for the indicated time periods and mixed at time zero with 3A2-opsonized YP137 (MOI = 50:1). Chemiluminescence was measured as described above. Cam, incubation in the presence of 100 μg of chloramphenicol per ml.

sion were prewarmed at 37°C for 5 min, mixed with 0.07 ml of IgG-coated YP137 (at a multiplicity of infection [MOI] of 50:1), and placed immediately in a luminometer (GEM Opticomp 1; MGM Instruments, Inc.). Chemiluminescence was measured for 2-s intervals every minute for a total of 10 min. As shown in Fig. 1A, chemiluminescence was detected within 3 min of mixing, and it increased rapidly over time, reaching a maximum of over 6,000 light units by 10 min. Addition of the antibody alone to the macrophages did not promote chemiluminescence (Fig. 1A), indicating that cross-linking of Fc receptors with opsonized *Y. pseudotuberculosis* was required for the generation of the oxidative burst. When macrophages were mixed with unopsonized YP137, chemiluminescence was low but detectable, reaching a maximum of 500 light units by 10 min (Fig. 1A). Although opsonized YP137 bound to the macrophages at a slightly higher level than YP137 (2-fold [Table 1]), this difference could not explain the >10-fold difference in chemiluminescence. In addition, opsonization of YP137 with mouse or guinea pig complement did not trigger an oxidative burst (data not shown). These results provided support for the idea that integrin-mediated interaction of *Y. pseudotuberculosis* with macrophages allows for evasion of bactericidal functions (13, 32). In addition, chemiluminescence was not observed when YP137 was grown at 37°C and incubated with the 3A2 antibody (data not shown). Since invasin expression in *Y. pseudotuberculosis* decreased at 37°C (31), it was likely that a high density of invasin epitopes on the bacterial surface was required to achieve effective opsonization and cross-linking of macrophage Fc receptors.

TABLE 1. Adherence of *Y. pseudotuberculosis* strains to macrophages

Strain[a]	Incubation with 3A2[b]	% Adherence[c]
YP202 (*inv*::*kan*)	−	2.7 ± 0.07
YP202 (*inv*::*kan*)	+	2.8 ± 0.3
YP137 (*inv*$^+$)	−	5.1 ± 0.3
YP137 (*inv*$^+$)	+	11.9 ± 1.2

[a] YP202 is derived from YP137 and does not express functional invasin (30).
[b] +, preincubation of the bacteria with 20 μl of hybridoma supernatant containing the 3A2 monoclonal antibody that is specific for invasin.
[c] Adherence was quantitated by mixing 5×10^7 bacteria with 1×10^6 suspended macrophages (MOI = 50:1) in 1 ml of HBSS on ice. Binding was allowed to occur at 4°C to prevent bacterial killing by the oxidative burst. The suspended cells were then seeded into the wells of a tissue culture plate and incubated on ice for 15 min to allow for adherence of the macrophages to the plastic. The wells were then washed three times with ice-cold HBSS (1 ml each). After washing, 1% Triton X-100 (0.2 ml) was added to lyse the macrophages, and bacterial CFU were quantitated by plating serial dilutions. Results are expressed as the percentages of bound bacteria relative to bacteria added (± standard deviations). Assays were preformed in triplicate.

Having determined that attachment of *Y. pseudotuberculosis* to macrophage Fc receptors was necessary to promote the oxidative burst, the next step was to measure the effect, if any, of YopH on this process. However, since YopH expression requires bacterial growth at 37°C, effective opsonization of the bacteria with the 3A2 antibody was not readily obtained. Therefore a mixed infection strategy was used to overcome this barrier. Macrophages were mixed sequentially with the wild-type *Y. pseudotuberculosis* strain, YP126 (42), which was grown at 37°C to express YopH, and YP137, which was grown at 26°C and opsonized with 3A2 to cross-link Fc receptors. Bacteria were prepared as described above, except that YP126 was grown at 37°C in LB supplemented with 2.5 mM $CaCl_2$. Under these conditions, the Yops are synthesized and localized to a detergent-insoluble membrane fraction, but they are not exported (14). A 0.05-ml volume of YP126 was mixed with 1.0 ml of suspended macrophages (MOI = 50:1) and placed at 37°C to allow for YopH expression. At various times thereafter (0, 1, 5, 10, and 15 min), the macrophages were mixed with IgG-coated YP137 to cross-link Fc receptors, and the production of an oxidative burst was assayed by chemiluminescence over a 10-min period. As shown in Fig. 1B, there was no major effect on chemiluminescence when Fc receptors were cross-linked immediately after the addition of YP126. In this case, chemiluminescence was detected at 3 min and reached a maximum of just over 5,000 light units by 10 min. However, significant decreases in chemiluminescence were observed when the macrophages were preincubated with YP126 for increasing lengths of time prior to Fc receptor cross-linking. For example, with 1 min of preincubation, the rate of chemiluminescence was unaffected; however, the maximal level reached only 4,000 light units. With 5 min of preincubation, both the rate and extent of chemiluminescence were reduced. At the extreme, chemiluminescence was minimal when the macrophages were incubated with YP126 for 15 min prior to Fc receptor cross-linking. Since inhibition of the oxidative burst by YP126 was due to expression of YopH (see below), the kinetics of this process indicated that the protein was rapidly exported and translocated into the macrophage, at least within 10 min of bacterial-host cell interaction.

Goguen et al. (20) have shown that protein synthesis is not required for export or translocation of YopE into macrophages when *Y. pestis* is grown at 37°C in medium containing 2.5 mM $CaCl_2$. In addition, protein synthesis is not required for export of invasion proteins by a homologous system in *Salmonella typhimurium* (19, 36). To determine if protein synthesis was required for *Y. pseudotuberculosis* to inhibit the oxidative burst, YP126 was mixed with macrophages (MOI, 50:1) in the presence of 100 μg of chloramphenicol per ml (20) for 15 min, and chemiluminescence was measured after cross-linking of Fc receptors (Fig. 1B). The results were consistent with the idea that Yop export and translocation are controlled at the posttranslational level, since YP126 inhibited the oxidative burst in the presence of chloramphenicol and chloramphenicol addition by itself did not inhibit chemiluminescence (data not shown).

The construction and analysis of a *yopH* insertion mutant of YP126 provided evidence that YopH was important for inhibition of the oxidative burst. A 2.8-kb *Pst*I restriction endonu-

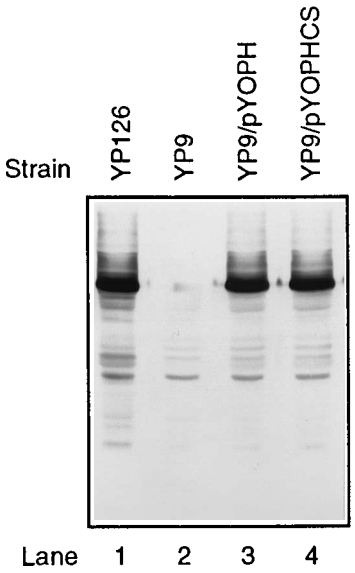

FIG. 2. Analysis of YopH expression in *Y. pseudotuberculosis* strains by Western blotting. The indicated *Y. pseudotuberculosis* strains were grown to mid-log phase in the presence of 2.5 mM Ca^{2+}. Equivalent amounts of total cellular protein from each strain were resolved on an SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody specific for YopH. The major bands detected in lanes 1, 3, and 4 correspond to YopH.

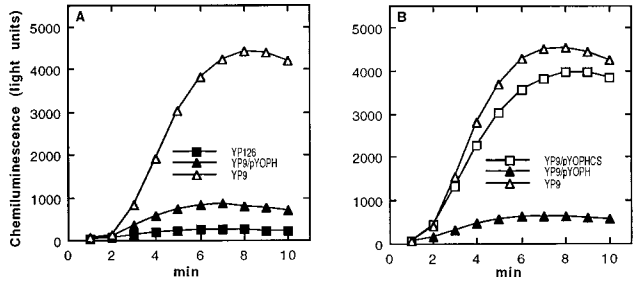

FIG. 3. (A) Effect of a *Y. pseudotuberculosis yopH* insertion mutant on inhibition of macrophage chemiluminescence. Macrophages were preincubated with the indicated strains for 15 min (MOI = 50:1) and then mixed at time zero with 3A2-opsonized YP137 (MOI = 50:1). Chemiluminescence was measured as described in the legend to Fig. 1. (B) Effect of the C403S catalytic mutation on inhibition of macrophage chemiluminescence by YopH. Macrophages were preincubated with the indicated strains for 15 min (MOI = 50:1) and mixed at time zero with 3A2-coated YP137 (MOI = 50:1). Chemiluminescence was measured as described in the legend to Fig. 1.

clease fragment of virulence plasmid DNA encompassing the *yopH* gene was inserted into the vector pFSV (5). The internal *Xba*I-to-*Kpn*I fragment (coordinates 459 to 916) of the *yopH* gene was then replaced with a 1.3-kb fragment derived from pDPT270 (40), which encodes resistance to chloramphenicol (*cam*). The mutated gene (*yopH*::*cam*) was introduced into the YP126 virulence plasmid using allelic recombination (5). The effect of the mutation on expression of YopH was examined by Western blotting (immunoblotting). Cultures of YP126 and YP9 (*yopH*::*cam*) were grown at 37°C in LB supplemented with 2.5 mM $CaCl_2$ to an OD_{600} of 0.5. Bacteria were centrifuged and resuspended to an OD_{600} of 1.0 in HBSS. One milliliter of the bacterial suspension was centrifuged, and the cell pellet was solubilized in 100 μl of Laemmli sample buffer (25). Proteins were fractionated on sodium dodecyl sulfate (SDS)-12% polyacrylamide gel electrophoresis (PAGE) gels and transferred to a nitrocellulose membrane. The membrane was incubated with antibody specific for YopH (RAY51) and goat anti-rabbit IgG-alkaline phosphatase conjugate (3). Antibody binding was visualized by incubating the membrane in 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (25). As shown in Fig. 2, this analysis confirmed that YP9 did not express intact YopH protein. Cultures of YP126 or YP9 were preincubated with macrophages for 15 min, and chemiluminescence was measured after the addition of IgG-coated YP137. There was minimal chemiluminescence in the presence of YP126 and significant chemiluminescence in the presence of YP9, indicating that YopH function was necessary for inhibition of the oxidative burst (Fig. 3A).

An expression plasmid carrying the *yopH* gene was constructed for complementation studies. PCR was used to amplify a DNA fragment containing the *yopH* open reading frame flanked by restriction sites. The sequences of the forward and reverse primers were 5′-CGGATCCACATATGAACTTAT CATTAAGC-3′ and 5′-GTCGGATCCTGAATTCGAATA AATATTTACATTAGC-3′, respectively. The *Bam*HI and *Nde*I restriction sites in the forward primer and the *Eco*RI restriction site in the reverse primer are underlined. Using the *Bam*HI and *Eco*RI restriction sites, the amplified DNA fragment was inserted into pBluescript II SK$^-$ (Stratagene), and the *yopH* gene was sequenced (Sequenase; U.S. Biochemical). Several discrepancies between this sequence and the published sequence of *yopH* (7) were noted. Sequencing of an independent copy of *yopH* indicated that the published sequence was in error because of compression artifacts. In the wild-type gene, codons 150, 151, and 211 are CTG, CGT, and GCA, respectively. These changes have been deposited in GenBank. The *yopH* gene was isolated from the pBluescript II SK$^-$ vector and inserted into pT7-7 (49) using the *Nde*I and *Eco*RI restriction sites. Expression of full-length YopH under control of the phage T7 RNA polymerase and T7 ϕ10 promoter was confirmed by SDS-PAGE (49). A DNA fragment consisting of *yopH* and the upstream ribosome-binding site in the pT7-7 vector was then isolated and inserted into pMMB67HE (17). In this way, *yopH* transcription was placed under control of the *tac* promoter and *lacI*^{q} repressor. The resulting plasmid, which also encoded resistance to ampicillin, was designated pYOPH. pYOPH was mobilized from SM10λ*pir* (37) into YP9 by mating and selection for resistance to ampicillin and chloramphenicol. Analysis of YP9(pYOPH) protein extracts by Western blotting showed that YopH was expressed constitutively from pYOPH at native levels without induction (Fig. 2, lane 3). In the chemiluminescence assay, YP9(pYOPH) was almost as effective as YP126 at inhibiting the oxidative burst (Fig. 3A), indicating that YopH was sufficient for inhibition of the oxidative burst.

Most tyrosine phosphatases have a conserved domain of approximately 300 amino acids which is responsible for catalysis. In YopH, this domain corresponds to amino acids 206 to 468 (11). A conserved Cys residue at position 403 has been shown to be essential for catalysis, since mutation of this reside to either Ser (C403S) or Ala (C403A) resulted in a total loss of enzymatic activity (22). Analysis of a *Y. pseudotuberculosis* YopHC403A mutant in an mouse infection model indicated that tyrosine phosphatase activity was also essential for pathogenicity (4). A derivative of pYOPH carrying the C403S mutation (pYOPHC403S) was introduced into YP9. After confirming expression of the mutant protein (Fig. 2, lane 4), a chemiluminescence assay was performed. As shown in Fig. 3B, tyrosine phosphatase activity was necessary for inhibition of the macrophage oxidative burst. Similar results were obtained

with the *Y. pseudotuberculosis* YopHC430A mutant (data not shown).

Since YopH produced by infection with wild-type bacteria was able to inhibit the oxidative burst triggered by secondary infection with opsonized bacteria, our results indicate that the tyrosine phosphatase activity of YopH inhibits Fc receptor-mediated signal transduction in macrophages in a global fashion. This finding suggests that YopH is capable of diffusing to, or is targeted to, the sites underneath the phagocytic cups where the signals are generated. The earliest steps in Fc receptor signalling in mouse macrophages involve the activation of Src-related tyrosine kinases and the phosphorylation of the γ subunit of Fc receptors (21). We envision that the negative influence of tyrosine phosphatase activity on the oxidative burst results from the dephosphorylation of these or other proteins in the Fc receptor signal transduction pathway. This activity appears to be somewhat specific for Fc receptor signal transduction pathways, since YopH did not inhibit activation of the oxidative burst triggered by binding of zymosan to macrophages (26) or the binding of complement-opsonized *Y. enterocolitica* to neutrophils (10).

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