

Yersinia pestis YopJ Suppresses Tumor Necrosis Factor Alpha Induction and Contributes to Apoptosis of Immune Cells in the Lymph Node but Is Not Required for Virulence in a Rat Model of Bubonic Plague

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Received 8 February 2006/Returned for modification 23 March 2006/Accepted 2 June 2006

The virulence of the pathogenic *Yersinia* species depends on a plasmid-encoded type III secretion system that transfers six Yop effector proteins into host cells. One of these proteins, YopJ, has been shown to disrupt host cell signaling pathways involved in proinflammatory cytokine production and to induce macrophage apoptosis in vitro. YopJ-dependent apoptosis in mesenteric lymph nodes has also been demonstrated in a mouse model of *Yersinia pseudotuberculosis* infection. These results suggest that YopJ attenuates the host innate and adaptive immune response during infection, but the role of YopJ during bubonic plague has not been completely established. We evaluated the role of *Yersinia pestis* YopJ in a rat model of bubonic plague following intradermal infection with a fully virulent *Y. pestis* strain and an isogenic *yopJ* mutant. Deletion of *yopJ* resulted in a twofold decrease in the number of apoptotic immune cells in the bubo and a threefold increase in serum tumor necrosis factor alpha levels but did not result in decreased virulence, systemic spread, or colonization levels in the spleen and blood. Our results indicate that YopJ is not essential for bubonic plague pathogenesis, even after peripheral inoculation of low doses of *Y. pestis*. Instead, the effects of YopJ appear to overlap and augment the immunomodulatory effects of other *Y. pestis* virulence factors.

The genus *Yersinia* includes three species that are pathogenic for humans and animals. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* cause food-borne and waterborne enteric disease, and *Yersinia pestis* causes plague, which is usually transmitted by flea bites. All three species harbor very similar ~70-kb virulence plasmids that encode a type III secretion system (TTSS), which functions to transport virulence proteins called Yops (*Yersinia* outer proteins) into eukaryotic cells (6). The six translocated Yop effector proteins (YopH, YopM, YopT, YopE, YpkA/YopO, and YopJ) interfere with mammalian cell signaling pathways, which inhibits phagocytosis, modulates cytokine production, and induces apoptosis (4, 16, 29). Current models of *Yersinia* pathogenesis postulate that the cumulative effect of the Yops and other proteins secreted by the TTSS attenuate the innate and adaptive immune responses (2, 5, 16, 29).

The plasmid-encoded TTSS is required for virulence of all three pathogenic *Yersinia* species (28), and biochemical functions of each of the effector Yops have been determined (for reviews see references 4, 16, and 29). However, the individual roles of the Yops during different stages of infection have not been completely established. For full virulence in mice, all

three *Yersinia* pathogens require both YopH and YopE, and *Y. pestis* and *Y. enterocolitica* require YopM (the role of YopM has not been determined yet for *Y. pseudotuberculosis*); however, loss of YopT has no effect on *Y. enterocolitica* virulence (this has not been tested yet for the other two species) (10, 11, 26, 27). Conflicting results have been reported for the effects of YpkA/YopO and YopJ on virulence. For example, loss of YopJ (called YopP in *Y. enterocolitica*) had no effect on *Y. pseudotuberculosis* infectivity in one study (9) but resulted in a 64-fold increase in the 50% lethal dose (LD₅₀) in another study (14). A *Y. enterocolitica* YopJ mutant was less infective after oral or intravenous challenge (27). In *Y. pestis*, insertional inactivation of *yopJ* increased the LD₅₀ only 1.2- to 1.5-fold in a septicemic plague model that included direct intravenous injection of an attenuated strain (25, 26).

YopJ has several biochemical functions that are predicted to impair the normal mammalian immune response to infection. YopJ is a deubiquinating cysteine protease that blocks activation of members of the mitogen-activated protein kinase family, including MKK and IKK β , thereby altering normal cytokine production and host cell survival pathways (1, 12, 17, 19, 22, 23, 33). These biochemical activities inhibit production of the proinflammatory cytokines tumor necrosis factor alpha (TNF- α) and interleukin 8 and induce macrophage apoptosis in vitro (13, 15, 23, 32). To determine whether these immunomodulatory activities occur in vivo and their role in the pathogenesis of plague, we deleted *yopJ* from the fully virulent *Y. pestis* 195/P strain and assessed the virulence of the resulting mutant after intradermal (ID) inoculation in a rat model of bubonic plague.

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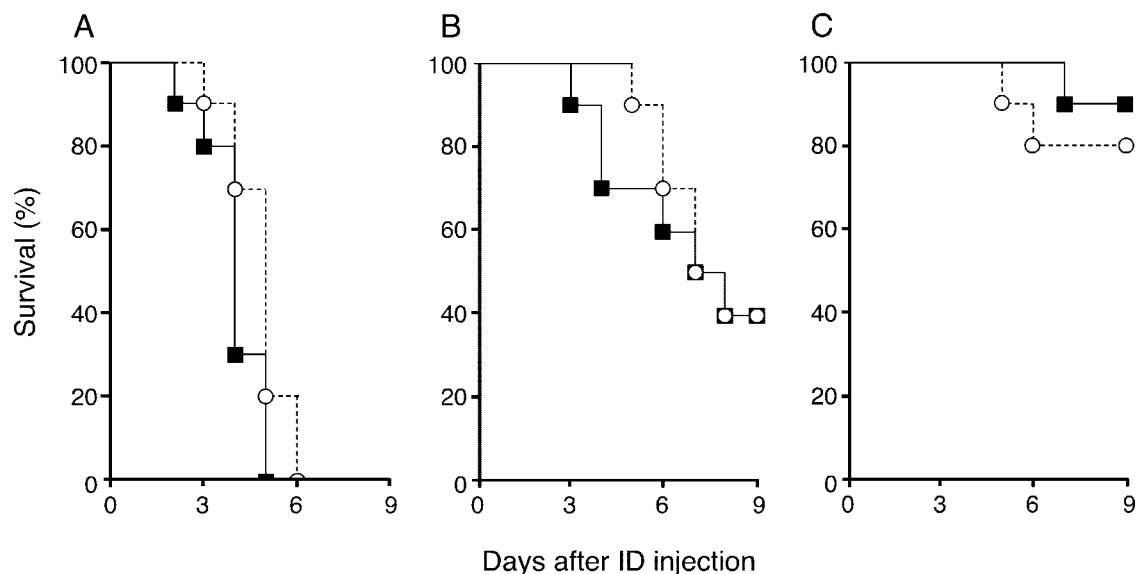


FIG. 1. Incidence of terminal plague in Brown Norway rats following intradermal injection of 100 CFU (A), 10 CFU (B), or ~1 CFU (C) of wild-type (solid squares) or $\Delta yopJ$ (open circles) *Y. pestis*. The calculated LD_{50} of both strains is <10 CFU.

MATERIALS AND METHODS

Construction of a *Y. pestis yopJ* mutant. An in-frame *yopJ* mutant of *Y. pestis* 195/P was constructed by allelic exchange mutagenesis. A 2-kb region encompassing the entire 867-bp *yopJ* coding sequence and upstream and downstream flanking regions was PCR amplified using primers YopJ1 (5'-CAGGAGGTATCGGAGTTAC-3') and YopJ2 (5'-GATCAGCGATGAGATGCTG-3') and cloned in pCRII-Topo (Invitrogen, Carlsbad, CA). Inverse PCR of the resulting plasmid with primers YopJ3 (5'-CAGGGAATTAACAGCGGTAT-3') and YopJ4 (5'-GGAATTATCAGTTCGGTAC-3') and religation yielded pCRII-Topo containing the *yopJ* sequence with an 803-bp internal deletion. The $\Delta yopJ$ allele was then subcloned into the suicide vector pCVD442 (7), which was transformed into *Escherichia coli* S17-1 λ pir. The *yopJ* deletion was introduced into the *Y. pestis* 195/P strain by conjugation and allelic exchange (7). The 803-bp internal deletion of *yopJ* in the *Y. pestis* $\Delta yopJ$ strain was verified by PCR and by Western blotting with a mouse anti-YopJ monoclonal antibody (data not shown).

Infection of rats and virulence testing. Wild-type *Y. pestis* 195/P and the isogenic $\Delta yopJ$ mutant were cultured overnight in brain heart infusion broth at 21°C, quantified by Petroff-Hausser direct counting, and diluted in phosphate-buffered saline (PBS) (pH 7.4). The numbers of *Y. pestis* CFU in the dilutions were checked by plating samples on *Yersinia* selective agar (Difco).

Groups of 10 8-week-old female Brown Norway rats (Charles River Laboratories, Wilmington, MA) were inoculated intradermally with 50 μ l of PBS containing ~1, 10, 100, or 1,000 CFU in the left upper anterior thigh. When signs of terminal plague sepsis appeared (roughcast fur, watery eyes, tachypnea, hunched posture, reluctance to move, and limping of the hind leg adjacent to the bubo [24]), rats were euthanized, and the inguinal lymph node (ILN) proximal to the injection site, spleen, and heart blood were aseptically collected. Spleens were weighed and bisected; one half of each spleen was then formalin fixed, and the other half was triturated in PBS. Triturated spleen tissue and 50 μ l of whole blood were diluted in PBS and plated on *Yersinia* selective agar to count CFU. The remaining whole blood was allowed to coagulate to obtain serum. All experiments were performed at biosafety level 3 and were approved by the NIH NIAID Rocky Mountain Laboratories biosafety and animal care and use committees in accordance with National Institutes of Health guidelines.

Histology and immunohistochemistry. Formalin-fixed spleen and ILN samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Apoptotic cells in additional ILN sections were detected by immunohistochemical staining using anti-caspase-3 polyclonal antibody CP229B (BioCare Medical, Concord, CA) (24). Caspase-positive cells in 20 random nonoverlapping microscopic fields (magnification, $\times 1,000$) were counted for ILN sections from seven rats infected with wild-type *Y. pestis*, eight rats infected with $\Delta yopJ$ *Y. pestis*, and four uninfected negative control rats.

Serum TNF- α assay. The TNF- α concentration in each serum sample collected at the terminal stage of disease was determined by enzyme-linked immu-

nosorbent assays using a rat BD OptEIA set (BD Biosciences, San Diego, CA) (24).

Statistical analysis. The LD_{50} was calculated by using the Reed-Muench equation, and differences in percentages of survival were evaluated by the log rank test. Correlations between infectious dose, time to terminal disease, bacterial loads in the spleen and blood, serum TNF- α level, and the number of ILN caspase-3-positive cells were calculated by using linear regression and the Pearson correlation. Differences between the mean numbers of ILN caspase-3-positive cells and serum TNF- α levels of rats infected with the two *Y. pestis* strains were evaluated by a two-tailed *t* test; a *P* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

***Y. pestis yopJ* mutant is fully virulent in a rat model of bubonic plague.** In the Brown Norway rat, intradermal injection of *Y. pestis* leads to bubonic plague (24). Within 72 h, the bacteria disseminate from the ID injection site through afferent lymphatic vessels to the regional draining lymph nodes. *Y. pestis* multiplies rapidly in a lymph node and produces severe lymphadenitis, which is characterized by extensive necrosis, hemorrhage, fibrin deposition, and large masses of densely packed extracellular bacteria throughout the node, associated with an edematous, hemorrhagic periadenitis (24). The enlarged infected lymph node is referred to as a bubo, and the same histopathology is seen in human bubonic plague (3). Systemic spread from the bubo leads to septicemic plague, which is characterized by high-density bacteremia and colonization of the spleen, liver, and other tissues.

To evaluate the effect of YopJ on the different stages and kinetics of the progression of bubonic plague, which may also include an initial intracellular stage in dermal phagocytes (20), we infected rats with a fully virulent *Y. pestis* strain and with the isogenic $\Delta yopJ$ mutant. The LD_{50} of both strains following ID injection was <10 CFU (Fig. 1). The survival rate, the time to terminal disease, and the progression of the clinical signs of plague were also the same for rats infected with the wild-type strain and rats infected with the mutant. The median times to

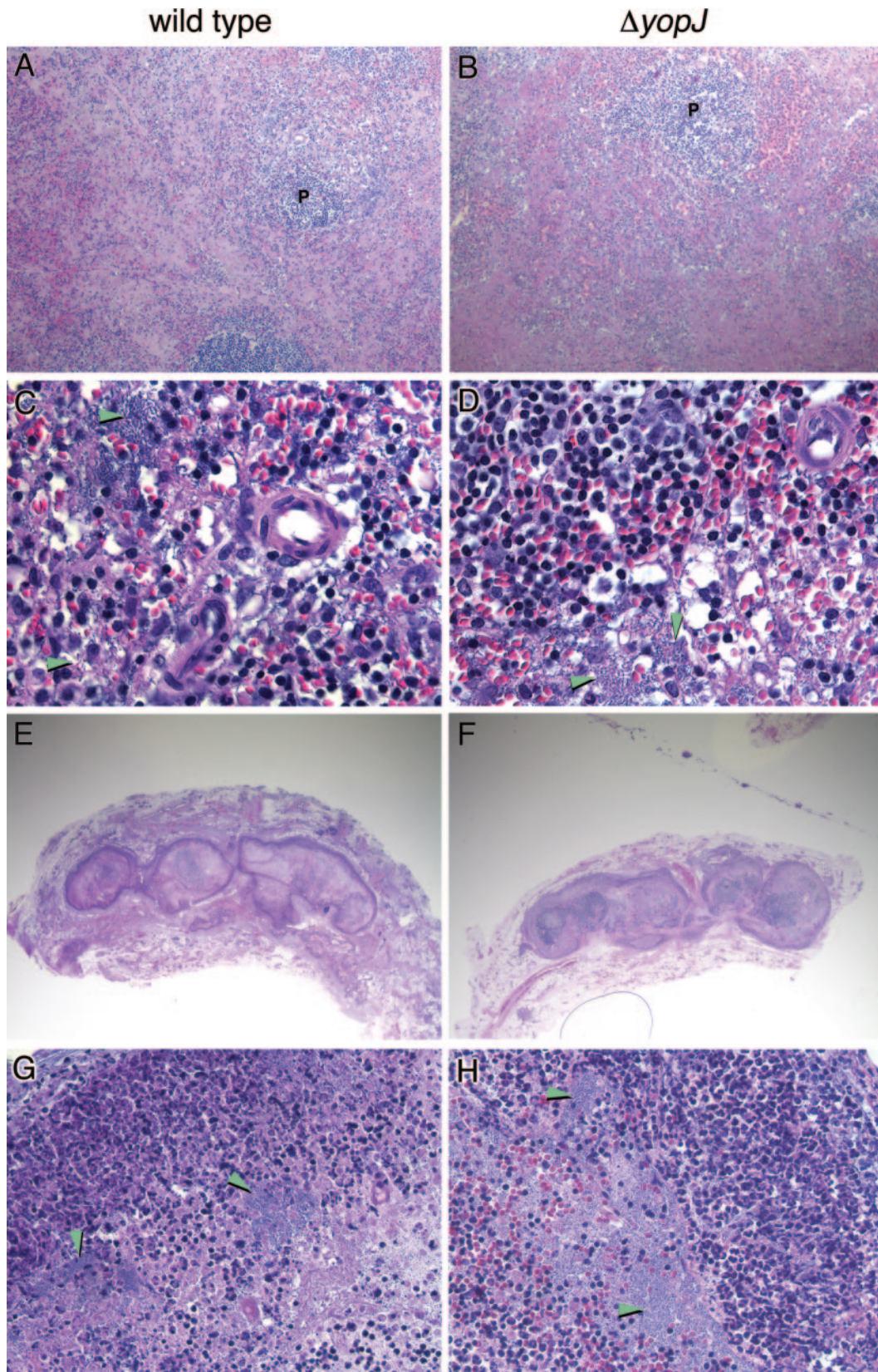


FIG. 2. Identical histopathologies produced by wild-type and $\Delta yopJ$ *Y. pestis*. Hematoxylin-and-eosin-stained sections of spleens (A to D) and inguinal buboes (E to H) from rats infected with wild-type (left column) or $\Delta yopJ$ (right column) *Y. pestis* were examined. The arrowheads indicate large aggregates of extracellular bacteria. P, periarterial lymphatic sheath. Original magnifications, $\times 200$ (A and B), $\times 600$ (C and D), $\times 10$ (E and F), and $\times 400$ (G and H).

terminal disease for rats infected with 100 CFU of the wild type and rats infected with 100 CFU of $\Delta yopJ$ *Y. pestis* were 4 and 5 days, respectively, and the maximum survival time was 6 days (Fig. 1A). The survival curves for rats infected with 100 or 1,000 CFU of the strains did not differ significantly. At a lower ID inoculation dose (10 CFU), 60% of the rats infected with either the wild-type or $\Delta yopJ$ strain developed plague, and the median time to terminal disease was 7.5 days (Fig. 1B). For each of the four infectious doses, the differences in incidence and the time to terminal disease between rats infected with the *yopJ* mutant and rats infected with the wild-type parent strain were not significant ($P > 0.05$). These results showed that *yopJ* is dispensable during all stages of the development of bubonic and septicemic plague.

In previous studies of the role of YopJ in *Y. pestis* pathogenesis the workers used attenuated strains lacking the *Yersinia* high-pathogenicity island that is required for virulence when peripheral routes of infection are used (25, 26). Direct intravenous injection of these strains indicated that YopJ is not required for the development of fatal septicemic plague in mice, but the results did not address the role of YopJ during earlier disease stages when the normal intradermal route of infection was used. Our results corroborate and extend the results obtained with the septicemic plague model and establish that YopJ is not required for virulence regardless of the dose or the route of infection.

Loss of YopJ does not affect systemic spread, colonization, or histopathology of bubonic plague. All rats that developed terminal plague with either the *Y. pestis* wild-type strain or the $\Delta yopJ$ strain had the same clinical signs, including a palpable enlarged inguinal bubo. After euthanasia, ILN and spleens were collected from all of these rats, and similar histopathologies were seen in both bubo and spleen tissue. The architecture of the ILN and spleen was markedly affected, and there were many large aggregates of extracellular bacteria and associated necrosis (Fig. 2). The bacterial loads in the spleen and blood were also similar (Fig. 3). Although the time to terminal disease was indirectly correlated with the initial ID injection dose (1, 10, or ≥ 100 CFU) (Fig. 1), the histopathologies of the bubo and the bacterial loads in the spleen and blood at the terminal stage of disease were equivalent for all rats, regardless of the strain or infectious dose (Fig. 2 and 3). The mean levels recovered were $10^{8.2}$ CFU/g spleen for all wild-type infected rats and $10^{7.8}$ CFU/g spleen for all $\Delta yopJ$ -infected rats. The mean bacteremia levels were $10^{7.3}$ CFU/ml for rats infected with the wild-type strain and $10^{6.2}$ for rats infected with the $\Delta yopJ$ mutant. The differences were not statistically significant ($P = 0.37$ and 0.09 , respectively).

***Y. pestis* YopJ contributes to apoptosis and suppresses TNF- α induction during bubonic plague.** Because YopJ appeared to have no effect on disease progression, virulence, histopathology, or systemic spread and tissue colonization, we sought evidence for two of its predicted functions, apoptosis of immune cells in the lymph node and suppression of the normal TNF- α response, during bubonic plague.

At the terminal stage of disease, the mean number of apoptotic cells in the bubo was two- to threefold higher in rats infected with wild-type *Y. pestis* than in rats infected with the $\Delta yopJ$ mutant ($P < 0.0001$) (Table 1). However, the number of apoptotic cells in $\Delta yopJ$ -infected rats was significantly higher

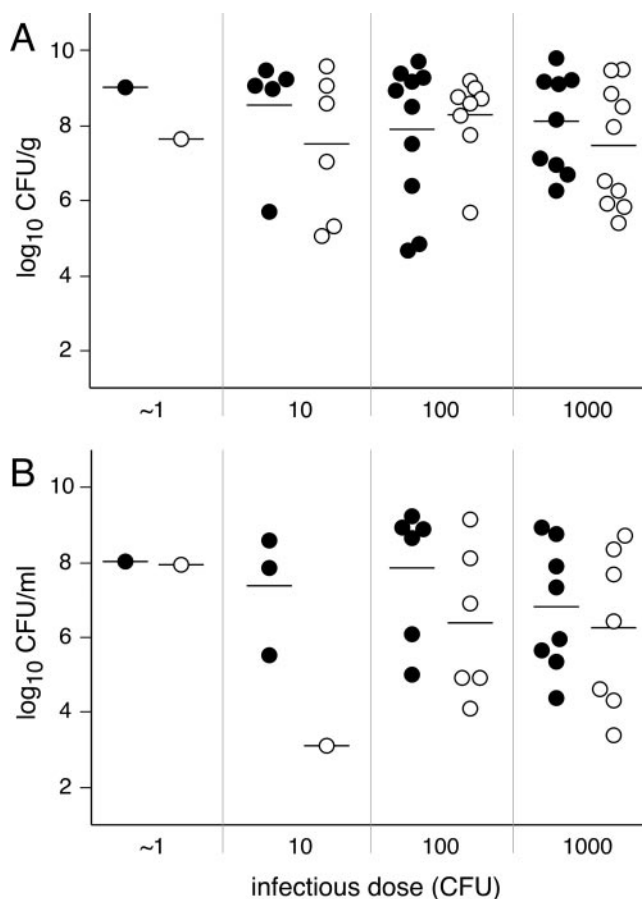


FIG. 3. Colonization in the spleen (A) and blood (B) in rats inoculated intradermally with ~1, 10, 100, or 1,000 CFU wild-type *Y. pestis* (solid circles) or $\Delta yopJ$ *Y. pestis* (open circles). Each symbol indicates the bacterial load in a single rat at the terminal stage of plague; the horizontal lines indicate the mean log CFU per gram of spleen or milliliter of blood.

than the background level (< 1 caspase-positive cell/field) in the ILN of uninfected controls. Thus, the biochemical activities of YopJ augment apoptosis of lymph node cells during bubonic plague but are not solely responsible for it.

The $\Delta yopJ$ mutant was also defective in the ability to suppress host TNF- α production during infection. The mean serum TNF- α level in rats with terminal plague caused by the $\Delta yopJ$ mutant was significantly higher ($P = 0.02$) than the level in rats infected with the wild type (Table 2). The TNF- α levels in the rats infected with the wild type are consistent with the results of a previous study (24), in which serum TNF- α was detected only in the late septic stage of disease. Again, although the presence of YopJ reduced the TNF- α level threefold, production of this cytokine was not completely suppressed, since all four uninfected control rats had < 40 pg/ml of serum. Additionally, there was no correlation between the numbers of CFU in the spleen or blood and the TNF- α levels or numbers of caspase-3-positive cells in rats infected with either wild-type or $\Delta yopJ$ *Y. pestis* ($P > 0.3$).

Our results indicate that although YopJ contributes to apoptosis and suppression of TNF- α during bubonic plague, it is not necessary for normal plague pathogenesis in rats. In contrast,

TABLE 1. Effect of *Y. pestis* YopJ on apoptosis in the bubo

Rat	Bacterial load in ^a :		No. of apoptotic cells/field ^a
	Blood (log CFU/ml)	Spleen (log CFU/g)	
Rats infected with <i>Y. pestis</i> wild type			
1	6.1	6.5	15.4
2	ND ^b	ND	15.4
3	ND	9.3	14.4
4	8.6	9.5	21.2
5	8.7	9.8	20.0
6	9.0	9.4	19.2
7	9.3	9.0	16.6
Mean ± SD			17.5 ± 2.6
Rats infected with <i>Y. pestis</i> Δ <i>yopJ</i>			
1	5.0	ND	7.5
2	ND	7.1	5.4
3	ND	7.8	4.6
4	ND	8.3	8.6
5	ND	8.8	8.9
6	8.2	8.8	7.4
7	ND	9.1	5.9
8	ND	9.2	5.7
Mean ± SD			6.8 ± 1.6 ^c

^a Mean number of caspase-positive cells in 20 random microscopic fields.

^b ND, not determined.

^c Statistically significantly different from the value for rats infected with *Y. pestis* wild type ($P < 0.0001$).

loss of *yopJ* in enteropathogenic *Yersinia* has been correlated with reduced bacterial persistence and virulence in mouse infection models. For example, Monack et al. showed that the number of cells of a *Y. pseudotuberculosis yopJ* mutant in the spleen decreased 100-fold by 3 days after oral infection, and the LD₅₀ of this mutant was 64-fold higher than that of the parental strain (14). Similarly, a *Y. enterocolitica yopP* mutant was slightly attenuated in an oral infection model because it was less able to colonize the small intestine and Peyer's patches and to cause systemic disease (27). On the other hand, Galyov et al. reported that YopJ had no effect on *Y. pseudotuberculosis* virulence or spleen colonization (9). These conflicting results could have been due to the different oral challenge doses given or to unrecognized genetic differences between the *yopJ* mutants or mouse strains used.

Y. pestis causes apoptosis of immune cells in an infected bubo (24). Our results indicate that the activity of YopJ does not entirely account for this. To date, YopJ is the only Yop effector known to induce apoptosis. However, lipopolysaccharide activation of Toll-like receptor 4-dependent pathways may be the primary stimulus for apoptosis, and YopJ may have a contributory role, inhibiting antiapoptotic pathways (21, 30, 31). Likewise, YopJ modulated but did not eliminate TNF- α induction in vivo, as predicted from in vitro studies with *Y. pseudotuberculosis* and *Y. enterocolitica yopJ/P* mutants (1, 17, 18, 21, 23). Nevertheless, neither the decreased apoptosis in the bubo nor the increased serum TNF- α level generated by the Δ*yopJ* mutant was sufficient to significantly delay systemic infection and the onset of fatal disease in rats (Fig. 1). Thus, YopJ appears to have a synergistic but nonessential role in *Y. pestis* pathogenesis. As is the case for the multiple TTSS ef-

TABLE 2. Effect of *Y. pestis* YopJ on TNF- α induction during bubonic plague

Rat	Bacterial load in ^a :		TNF- α concn (pg/ml serum)
	Blood (log CFU/ml)	Spleen (log CFU/g)	
Rats infected with <i>Y. pestis</i> wild type			
1	4.4	5.0	292
2	5.0	5.5	<40
3	5.4	4.9	1,105
4	6.1	7.1	<40
5	7.3	8.2	317
6	9.3	9.1	128
Mean ± SD			320 ± 403
Rats infected with <i>Y. pestis</i> Δ <i>yopJ</i>			
1	3.4	5.5	623
2	4.2	ND	411
3	5.0	ND	1,537
4	5.0	5.7	2,277
5	6.5	8.0	1,026
6	7.7	8.9	1,453
7	9.2	9.3	681
Mean ± SD			1,144 ± 655 ^b

^a ND, not determined.

^b Statistically significantly different from the value for rats infected with *Y. pestis* wild type ($P = 0.02$).

factors of *Salmonella*, however, any one of which is often dispensable for virulence in a given animal model (8), the *Y. pestis* Yops may constitute a multifactorial system whose combined effects ensure productive infection in a range of hosts.

ACKNOWLEDGMENTS

We thank David Erickson, Viveka Vadyvaloo, and Leigh Knodler for reviews of the manuscript. We also acknowledge Richard Siegel, NIH NIAMS, for his gift of an anti-YopJ monoclonal antibody.

This work was supported by the Division of Intramural Research, NIAID, NIH, and by the Ellison Medical Foundation (New Scholars Award in Global Infectious Diseases to B.J.H.).

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Editor: J. B. Bliska