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Role of YadA in Arthritogenicity of *Yersinia enterocolitica* Serotype O:8: Experimental Studies with Rats

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Outer membrane protein YadA, the *Yersinia* adhesin, is one of the plasmid-encoded virulence factors of yersiniae. To evaluate the role of YadA in the pathogenesis of reactive arthritis experimentally, we used YadA$^-$ strain YeO8-116, a kanamycin GenBlock insertion mutant derived from *Yersinia enterocolitica* O:8 wild-type strain 8081. As control strains, a plasmid-cured derivative (8081-c) of 8081 and a YopH$^-$ mutant (8081-yoph) were used. In addition, YeO8-116, with the *yadA* mutation transcomplemented with plasmid pMW10, was used. YeO8-116 induced arthritis to a considerably lesser extent than did wild-type strain 8081 when inoculated intravenously into Lewis rats. In rats surviving for over 14 days after the bacterial inoculation, the arthritis incidences were 6% (4 of 72) among those inoculated with the *yadA* mutant and 51% (33 of 65) among those inoculated with wild-type strain 8081. When the *yadA* gene was transcomplemented back to YeO8-116, YeO8-116/pMW10 induced arthritis in 47% (9 of 19) of the inoculated rats. Plasmid-cured strain 8081-c did not induce arthritis in any of the 24 inoculated rats, whereas YopH$^-$ mutant 8081-yoph induced arthritis in 20% (5 of 25) of the rats inoculated. Although the 50% lethal dose of YeO8-116 was about sixfold higher than that of 8081, the kinetics of bacterial elimination from the spleen and mesenteric lymph nodes were about the same with both strains. Antibody responses in rats infected with the two strains were also indistinguishable. Our results indicate that YadA contributes to the arthritogenicity of *Y. enterocolitica* in the rat model.

In studies on the pathogenesis of reactive arthritis, a crucial question is the demonstration and characterization of arthritogenic molecules of the causative microbes and their relation to host defense mechanisms. In the human disease, HLA-B27 or closely linked genes are important. In experimental models of *Yersinia*-associated arthritis in Lewis and SHR rats, the major histocompatibility complex seems not to be of importance, since rats of major histocompatibility complex-compatible control strains are not susceptible to induction of arthritis (12, 15, 23, 47). On the other hand, the experimental models offer a unique opportunity to search for the causative arthritogenic molecules (41).

In *Yersinia enterocolitica*, virulence is determined by several factors. Chromosomally encoded genes *inv* and *ail* are needed for virulence (16, 24). Invasin plays an important role in the first stage of infection by initiating bacterial entry into host cells by using fibronectin receptors (17, 29). A heat-stable enterotoxin, known as YST, and the O-side chain of lipopolysaccharide (LPS) are also important for virulence (1, 27). Animal studies with *yst* deletion mutants imply that this enterotoxin is crucial for diarrhea induction (10). Besides these chromosomally encoded factors, virulence plasmid pYV is responsible for several phenomena crucial for bacterial pathogenesis (reviewed by Straley et al. [44]). One of them is outer membrane protein YadA (yersinia adhesin A). YadA has been associated with host defense, reacting through numerous different mechanisms. It is largely responsible for the adhesion of *Y. enterocolitica* to intestinal tissue in vitro (21, 26) and attachment to and entry into HEp-2 cells in vitro (6, 14). The protein is also associated with bacterial autoagglutination (2, 42) and serum resistance (2, 9, 22, 31). YadA is able to bind factor H in serum, thus helping the bacteria to bypass complement activation (9, 40). China et al. (8) have seen a correlation between YadA expression and the reduction of phagocytosis and of killing by polymorphonuclear leukocytes. The protein is also responsible for inhibition of the anti-invasive effect of interferon (6). YadA is expressed as fibrils on the bacterial surface (19), mediating binding to collagen types I, II, III, IV, V, and XI (11, 38, 45), to laminin (45), and to fibronectin (37, 46). Because of the binding properties of YadA to extracellular matrix molecules, the present study was undertaken to clarify the role of YadA in the induction of *Yersinia*-associated experimental arthritis in rats.

MATERIALS AND METHODS

Rats. Adult male Lewis/SsNHsd rats weighing 225 to 275 g were purchased from Harlan Sprague Dawley, Inc., Indianapolis, Ind. Rat health was monitored by both the breeder and Microbiology Laboratories, North Harrow, Middlesex, England. The rats were found to be free of the following rat pathogens: (i) by serology, Hantaan virus, Kilham rat virus, *Mycoplasma pulmonis*, pneumonia virus of mice, reovirus type 3, Sendai virus, sialodacryoadenitis virus, and Toolan H-1 virus; (ii) by bacterial culture, *Bordetella bronchiseptica*, *Corynebacterium kutscheri*, *Mycoplasma* sp., *Pasteurella pneumotropica*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Streptobacillus moniliformis*, *Streptococcus pneumoniae*, and *Streptococcus zooepidemicus*. They were also found to be free of *Bacillus piliformis* when examined by diagnostic provocation. The rats were kept on autoclaved bedding of aspen wood in Macrolon cages under filter tops. The cages were in laminar-flow hoods, and the rats were fed a standard autoclaved diet and given water ad libitum.

Bacteria. The bacterial strains and plasmids used in this

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Comments	Source and/or reference
E. coli strains		
HB101	Δ(*gpt-proA*)*62 leuB6 thi-1 lacY1 hsdS*^{B}*20 recA rpsL20* (Str^{r}) *ara-14 galK2 xyl-5 mtl-1 supE44 mcrB*$_{B}$	5
S17-1	*thi pro hsd* (r^{-} m^{+}) *recA*::RP4-2-Tc::Mu-Km::Tn*7*	39
Sm10λpir	*thi thr leu tonA lacY supE pir* RK6 *recA*::RP4-2-Tc::Mu-Km^{r}	25
Y. enterocolitica strains		
WA	Serotype O:8, wild type	7
1881	Serotype O:3, patient isolate	33
8081	Serotype O:8, wild type, pYV^{+}	32
8081-c	pYV-cured derivative of 8081	32
YeO8-116	*yadA*::Km^{r}-GenBlock of 8081	18
8081R^{-}M^{+}	r^{-}m^{+} derivative of 8081	50
8081-yoph	*yopH*::Clm^{r}-GenBlock of 8081R^{-}M^{+}	This work
Plasmids		
pMW10	*yadA* of 8081 cloned into low-copy-number vector pCJ179, which carries *oriR* of pSC101 and *cat* of pACYC184	V. L. Miller; 30
pSFV7	Restriction fragment of *Y. pseudotuberculosis* YPIII/pIBI containing Clm^{r} cassette in *yopH* gene cloned into suicide vector pFSV	J. B. Bliska; 4

work are listed in Table 1. The bacteria were routinely stored at −70°C in Tryptone soy broth (TSB; Oxoid, Basingstoke, Hampshire, England) containing 20% glycerol. When appropriate, the following antibiotic concentrations were used in growth media unless otherwise indicated: kanamycin (KM), 20 μg/ml; chloramphenicol (CLM), 20 μg/ml; tetracycline (TET), 12 μg/ml. *Yersinia* strains were grown on lactose agar, Luria agar, or CIN agar (yersinia selective agar; Oxoid). *Escherichia coli* strains were grown on Luria agar plates.

Before each animal experiment, the *Yersinia* strains were recovered from −70°C by culturing at room temperature on lactose plates; YeO8-116 was cultured on Luria agar-KM plates. Expression of YadA was routinely verified by autoagglutination testing (20). For animal injections, bacteria were grown at room temperature on an orbital shaker (180 rpm) in 1 liter of TSB in a 2-liter Erlenmeyer bottle for 11 h; YeO8-116 was grown with KM (100 μg/ml), YeO8-116/pMW10 was grown with KM and CLM, and 8081-yoph was grown with CLM. Bacterial cells were collected by centrifugation, washed three times with 0.9% NaCl, and finally diluted to appropriate concentrations in 0.9% NaCl by using McFarland turbidity standards. The actual concentration of viable bacteria was determined by colony counting on agar plates. Suitable doses of bacteria were injected once into a tail vein in a total volume of 100 to 200 μl. During the injections, rats were lightly anesthetized with methoxyflurane (Metofane; Pitman-Moore, Washington Crossing, N.J.).

Construction of *Yersinia* strains. Standard recombinant DNA methods were used to manipulate DNA and construct strains. Construction of *yadA* mutant YeO8-116 will be described elsewhere (18). In brief, the *yadA* gene was inactivated by a KM resistance-encoding GenBlock insertion (Pharmacia, Uppsala, Sweden) located between the promoter and the open reading frame of the gene.

YadA expression in YeO8-116 was transcomplemented by introduction of pMW10 (a kind gift of Virginia L. Miller, University of California at Los Angeles), which carries an intact copy of the *yadA* gene of 8081 cloned into low-copy-number vector pCJ179 (Table 1). Plasmid pMW10 was mobilized into YeO8-116, and Km^{r} Clm^{r} transconjugants which carried both the mutated *yadA* gene and the transcomplementing wild-type *yadA* gene were first analyzed for YadA expression by using the autoagglutination test. Plasmid DNA was isolated from autoagglutination-positive clones, and the presence of pMW10 was checked by plasmid isolation and restriction fragment analysis. Before YeO8-116/pMW10 was used in animal experiments, the stability of the construct was confirmed. YeO8-116/pMW10 was cultured for several passages without antibiotic selection before the antibiotic resistance and plasmid profiles were reexamined. Both the antibiotic and plasmid profiles were found to be unaltered, implying that the construct was stable. Stability was also confirmed later by culturing the strain from stool samples of infected rats.

To construct a *yopH* mutant, we applied the marker exchange technique with plasmid pFSV-7, a kind gift from James B. Bliska (State University of New York, Stony Brook). Plasmid pFSV-7 is a suicide vector derived from pFSV (4) containing a 2.8-kb *Pst*I fragment of pIB1 (the virulence plasmid of *Y. pseudotuberculosis* YPIII/pIB1) encompassing the *yopH* gene. In pFSV-7, an approximately 450-kb *Xba*I-*Kpn*I fragment of *yopH* was replaced with a 1.3-kb CLM resistance-encoding cassette. pSFV-7 was mobilized into 8081-R^{-}M^{+} by conjugation. To select for clones with pFSV-7 integrated into the virulence plasmid by homologous recombination, bacteria were plated on CIN-CLM plates. Clm^{r} colonies were pooled and grown in Luria broth-CLM for three passages at 22°C. Cycloserine treatment was used to enrich for bacteria that had undergone a second recombination event, resulting in excision of both the suicide vector sequences and the wild-type *yopH* gene (28). The last overnight culture was diluted 1:20 into Luria broth containing CLM and tetracycline (20 and 12 μg/ml, respectively) and allowed to grow at 22°C for 2 h. Cycloserine was added to the culture to a final concentration of 2 mg/ml, and incubation was continued for another 2 h. During this treatment, clones that had undergone a second recombination event and were Tet^{s} did not grow while those still carrying the vector sequences did grow in the presence of tetracycline and were killed after addition of cycloserine. The enriched suspension was plated on CIN-CLM agar, and individual colonies were analyzed for loss of tetracycline resistance. The appropriate replacement of the wild-type *yopH* gene in one Clm^{r} Tet^{s} clone, designated 8081-yoph, was confirmed by isolation

of the virulence plasmid and performance of restriction fragment analysis with *Cla*I digestion (data not shown). The virulence plasmid-associated phenomena were not affected in 8081-yoph; it was calcium dependent and autoagglutination positive and secreted the Yop proteins, except YopH, normally.

Isolation of bacteria from rat tissues. Samples were taken aseptically from ankle joints, heart blood, mesenterial lymph nodes, and spleens of rats sacrificed 3, 7, 14, 21, 28, and 35 days after *Yersinia* injection. The samples were directly plated on lactose or KM plates and, for enrichment, inoculated into TSB (for YeO8-116, the TSB was supplemented with 100 μg of KM per ml) and incubated for 1 week at 4°C or room temperature. For quantitative studies, the spleen and lymph node samples were homogenized, diluted in TSB, and cultured. Results were reported as CFU of bacteria per gram of organ. *Y. enterocolitica* colonies were identified with API 20E strips (API System S.A., Montalieu, France) and by slide agglutination with serotype-specific rabbit antiserum.

Determination of *Y. enterocolitica*-specific antibodies. *Y. enterocolitica* serotype O:8 (YeO:8)-specific antibodies in rat sera were measured by enzyme immunoassay using formalin-killed and sodium dodecyl sulfate-treated strain 8081 as the antigen (12). Serum samples taken at different time points were randomized so that samples from YeO8-116-, 8081-, 8081-c, and 1881-injected and control rats were on the same microtiter plates.

Estimation of arthritis. Each limb was examined daily by two independent observers during the first 2 weeks after *Yersinia* inoculation. Later, the rats were examined every second day. A score of 0 to 4 was assigned for each limb, 0 standing for negative and 4 standing for gross distortion with severe arthritic changes (12, 47). The rats were monitored for 35 days after bacterial inoculation.

Assessment of histological changes. The hind feet were cut between the knee and ankle, removed, and fixed in 10% neutral buffered formalin. After fixation, they were decalcified, embedded in paraffin, cut on glass slides, and stained with hematoxylin and eosin. Histology scoring was performed by one person without knowledge of the specimen's identity.

MAb. For immunofluorescence (IF) staining, a murine YeO:8-specific (MAb) was used. To obtain the MAb, BALB/c mice were immunized every 2 weeks with YeO:8 strain WA, twice intraperitoneally in Freund's complete adjuvant, and once without adjuvant. Four days after the last injection, spleen cells from the injected BALB/c mice were fused with NS-1 mouse myeloma cells. The hybridoma clones obtained were tested by enzyme immunoassay for the presence of antibodies specific for YeO:8 by using whole YeO:8 bacteria and isolated YeO:8 LPS, for cross-reactivity with YeO:3 by using whole bacteria or isolated YeO:3 LPS, and for cross-reactivity with *E. coli* by using whole bacteria as the antigen. IF staining was used to test the actual MAb for specificity against YeO:3, YeO:8, and *E. coli*. One clone, Fu26-1F1, produced an immunoglobulin G1 (IgG1) class MAb specific for the O antigen of the LPS of YeO:8, as verified by immunoblotting. As a negative control, mouse IgG1 MAb Fu5-11G2, specific for a chicken bursa Bu-1b alloantigen (49), was used.

IF staining. For IF staining, tissue samples were taken aseptically at various time points from ankle synovia, mesenterial lymph nodes, and spleens. The specimens were immersed in 5% gelatin and frozen in liquid nitrogen. The tissue blocks were cut into 5-μm-thick sections with a cryomicrotome. The sections were fixed in acetone for 10 min in −20°C and incubated for 30 min at room temperature with the MAb. The bound antibody was stained with a $F(ab')_2$ rabbit anti-mouse IgG-fluorescein isothiocyanate conjugate adsorbed on a rat Ig affinity column to remove cross-reacting antibodies with rat Ig (Serotec, Oxford, England). The diluting buffer contained 5% normal rat serum to block nonspecific binding. The samples were examined with a fluorescence microscope by two independent observers unaware of the sample origin.

Tissue fixation and preparation for PCR. For bacterial detection by PCR, synovial samples were stored in Eppendorf tubes (12 samples) or in gelatin blocks (18 samples) at −70°C. For PCR, each sample was suspended in 200 μl of distilled water and heated at 94°C for 10 min. After cooling, proteinase K was added to a final concentration of 100 μg/ml and the tubes were incubated at 56°C overnight. The samples were extracted twice with phenol-chloroform-isoamyl alcohol and twice with ether.

Primers used for PCR. Universal bacterial oligonucleotide primers MS37 (5′-AGG ATG TTG GCT TAG AAG CAG CCA-3′) and MS38 (5′-TAA GGT AGC GAA ATT CCT TGT CGG G-3′) were used. The primers were constructed from the conserved regions of the 23S rDNA sequences and are specific for all of the eubacterial species studied thus far (43). To verify the presence of rat DNA in synovial samples, a collateral amplification was performed by using rat β-globin gene-specific primers RBG1 (5′-TAG CCA CCC TGA CTA GGT AT-3′) and RBG2 (5′-CAA CAT TAT CAG GGT TCA CC-3′). These primers were generated by using the published DNA sequence in the GenBank database (accession number X06701). All of the synovial DNA samples were positive with these primers, confirming the quality of the DNA extraction.

PCR conditions. Standard PCR amplification was performed as described by Saiki et al. (36), in 0.5-ml polypropylene tubes with a thermal reactor (Hybaid). The tubes contained 10 mM Tris-HCl (pH 8.8 at 25°C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, each deoxynucleoside triphosphate at 200 μM, 2 U of DynaZyme thermostable DNA polymerase (Finnzymes Oy, Espoo, Finland), 10 pmol of each primer, and 5 μl of the template. The total incubation volume was 50 μl. When primers MS37 and MS38 were used, the PCR steps were 94°C for 45 s, 60°C for 60 s, and 72°C for 120 s and were repeated 30 times. Under these reaction conditions, it was possible to detect fewer than 10 bacteria in the sample. The steps for the RBG primers were 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s and were repeated 35 times. To exclude the presence of inhibitory factors in the extracted DNA, parallel reactions were performed with tubes containing synovial DNA and purified YeO:8 DNA. The PCR products were detected with ethidium bromide and agarose gel electrophoresis.

RESULTS

Health status of inoculated rats. The six groups of rats, injected with strain YeO8-116, 8081, 8081-c, YeO8-116/pMW10, or 8081-yoph or 0.9% NaCl, were checked daily or every second day for general condition and development of arthritis. Rats injected with any of the bacterial strains were affected by the bacterial inoculation, whereas the NaCl controls remained healthy. The fur of the rats injected with bacteria was ruffled, the scrotums were red and swollen, and the paws were red and irritated. As a general observation at the autopsies, strain 8081 induced skin abscesses more often than did strain YeO8-116. Skin abscesses were found in 30 of 62 rats inoculated with strain 8081 and in only 8 of 76 rats inoculated with strain YeO8-116. A similar phenomenon has been seen in mice infected intragastrically with these two *Yersinia* strains (18). The symptoms caused by YeO8-116/pMW10 resembled those caused by wild-type strain 8081, including abscess for-

TABLE 2. Virulence and arthritogenicity of YeO:8 strains

Bacterial strain and dose	No. of rats			Mean arthritis score[a]	No. of days to death postinfection
	Injected	Dead	Arthritic		
YeO8-116					
3×10^9	4	4	0		1
6×10^8	4	2	0		5, 6
4×10^8	5	2	2	5.75	5, 10
3×10^8	4	2	0		1
2×10^8	5	0	0		
7×10^7	5	0	0		
4×10^7	34	0	2	0.75	
1×10^7	5	0	0		
8×10^6	13	0	0		
7×10^5	3	0	0		
8081					
1×10^8	6	4	0		1–5
7×10^7	6	5	0		2–15
5×10^7	12	0	9	5.33	
1×10^7	18	0	13	4.21	
8×10^6	5	0	4	2.90	
6×10^6	15	0	6	5.21	
2×10^6	5	0	1	1	
1×10^6	6	0	0		
8081-c					
3×10^9	5	5	0		1
1×10^9	5	0	0		
5×10^8	5	1	0		1
1×10^8	5	0	0		
4×10^7	5	0	0		
1×10^7	5	0	0		
YeO8-116/ pMW10					
4×10^8	5	5	0		1
1×10^8	5	5	0		1–4
4×10^7	5	0	3	3.42	
2×10^7	5	0	1	0.5	
1×10^7	5	0	3	1.58	
2×10^6	4	0	2	1.75	
8081-yoph					
7×10^8	3	2	0		1
2×10^8	5	0	1	2.25	
1×10^8	5	0	0		
7×10^7	5	0	2	1.25	
2×10^7	5	0	1	0.5	
1×10^7	5	1	1	0.5	1

[a] Only rats with arthritis included.

mation. Rats inoculated with strain 8081-c were the most healthy. Rats surviving endotoxin shock (the symptoms lasted, at most, for 2 days) recovered quickly, and no abscesses or other serious symptoms were noticed.

Virulence studies. To assess the virulence of the *Yersinia* strains studied, increasing doses of bacteria were administered intravenously into the tail vein. The least virulent strains were 8081-c and 8081-yoph, with an estimated 8081-c 50% lethal dose of around 2×10^9 bacteria (Table 2). Rats injected with 3×10^9 8081-c bacteria died within 24 h because of endotoxin shock, and rats injected with 1×10^9 bacteria were very sick for the first 2 days after inoculation but recovered quickly. One of the rats injected with the smallest dose of 8081-yoph also died within 24 h, most probably because of failed anesthetics. YeO8-116 was more virulent than 8081-c; YeO8-116 killed half of the animals inoculated with 3×10^8 to 6×10^8 bacteria, and the deaths occurred 1 to 10 days after the injections. YeO8-116 was, however, clearly less virulent than 8081, which had an estimated 50% lethal dose of around 6×10^7 bacteria. On the basis of results presented in Table 2, we estimated that YeO8-116 was about sixfold less virulent than 8081 by the intravenous route. The virulence of YeO8-116 returned to the wild-type level after introduction of transcomplementing plasmid pMW10 (Table 2).

Arthritogenicity. Among the rats surviving for over 14 days after the bacterial inoculation, 4 (6%) of the 72 injected with YeO8-116 developed arthritis. Two of the five rats surviving doses of $\geq 4 \times 10^8$ bacteria (higher than the estimated 50% lethal dose of this mutant) developed arthritis (Table 2). In one of them, the arthritis was confirmed by histology, whereas the other one macroscopically showed a mild arthritis (score, 1.5) not seen in histology at 2 weeks after the onset. In two other arthritic rats injected with 4×10^7 bacteria, the arthritic symptoms were mild, yielding a score of 0.75 for 3 days. Several of the animals had red and irritated paws, but histologic examination revealed no significant inflammation.

Thirty-three (51%) of the 65 surviving rats injected with strain 8081 developed arthritis. When sublethal doses were used, the arthritis incidence was highest (72 to 80% with doses of 5×10^7 to 8×10^6 bacteria; Table 2), and when smaller doses (6×10^6 and 2×10^6) were injected, it decreased (40 and 20%, respectively).

A total of 27 paws from strain 8081-injected rats and 26 paws from strain YeO8-116-injected rats, taken at different time points (7 to 35 days after the bacterial injection), were studied histologically. Histologic examination results were in good agreement with macroscopic findings. Clear inflammation was observed mainly in the synovia but also in the muscles surrounding the joints and in the cartilage. The inflammation consisted of edema with infiltrating lymphocytes; also, some neutrophils and macrophages were observed.

To confirm that the loss of the arthritogenetic potential of YeO8-116 was due to lack of YadA expression, an intact *yadA* gene was introduced into strain YeO8-116 in *trans*. Indeed, 9 (47%) of 19 rats infected with YeO8-116/pMW10 and surviving for ≥ 14 days developed arthritis, similar to those injected with strain 8081 (Table 2). This result is also quite similar to that obtained with wild-type strain 8081 regarding the arthritogenic doses of bacteria. As a further control, rats were injected intravenously with different doses of the $YopH^-$ mutant (8081-yoph), which expresses *yadA* but is less virulent than 8081. Of these, 25 rats survived for 14 days and 5 (20%) developed arthritis, which is clearly more than the 6% arthritis incidence seen after injection of YeO8-116 ($P = 0.046$; Fisher's exact test). The significance of this finding is emphasized by the fact that strain 8081-yoph was less virulent than strain 8081 (Table 2). Plasmid-negative strain 8081-c failed to induce arthritis in any of the 24 surviving rats.

Kinetics of infection. A number of rats injected with strain YeO8-116 or 8081 were sacrificed at different time points after injection. Samples from mesenterial lymph nodes and spleens were homogenized and cultured. No clear difference in kinetics between the two strains used was observed although YeO8-116 showed a tendency to be cleared from the spleen and mesenteric lymph nodes slightly earlier than 8081 (Table 3). By direct bacterial culture, small numbers of bacteria were isolated from the spleens and lymph nodes up to 14 days after injection. With enrichment, both strains were isolated, especially from the lymph nodes of some rats, at 28 and 35 days after injection (Table 3).

IF staining of spleen samples with a MAb specific for YeO:8 LPS revealed positively stained cells in almost all of the

TABLE 3. Infection kinetics of strains YeO8-116 and 8081 in lymph nodes and spleens

Bacterial strain, dose, postinfection day of sacrifice	Lymph node cultures		Spleen cultures	
	Direct[a]	Enriched[b]	Direct[a]	Enriched[b]
YeO8-116				
8.3×10^6				
3	1.5×10^4	ND[c]	2.5×10^3	ND
7	1.6×10^3	2/3	217	2/3
14	1.5×10^3	2/3	0	0/3
21	320	1/3	0	0/3
28	0	0/3	0	0/3
4.0×10^7				
3	3.2×10^4	3/3	2.5×10^3	3/3
7	2.2×10^3	3/3	455	3/3
14	0	1/3	0	1/3
21	0	1/3	0	1/3
28	1.9×10^3	1/3	0	0/3
35	0	0/5	0	0/3
4.5×10^7				
3	2.8×10^5	3/3	2.9×10^3	3/3
7	6.7×10^3	3/3	193	3/3
14	1.5×10^3	2/3	55	2/3
21	0	2/3	0	2/3
28	0	1/3	0	0/3
35	0	0/3	0	0/3
8081				
4.6×10^6				
3	1.3×10^3	ND	1.8×10^3	ND
7	9.2×10^6	3/3	231	3/3
14	0	0/3	170	1/3
21	0	0/3	0	0/3
28	0	0/3	0	0/3
1.1×10^7				
3	4.6×10^3	2/3	1.1×10^3	3/3
7	2.0×10^3	2/3	606	2/3
14	3.0×10^3	2/3	34	2/3
21	205	1/3	0	1/3
28	365	2/3	0	0/3
35	325	1/3	0	0/3
5.0×10^7				
3	4.2×10^4	3/3	2.9×10^3	3/3
7	3.0×10^3	2/3	604	3/3
14	1.9×10^3	3/3	0	3/3
21	0	1/3	91	2/3
28	0	0/3	30	1/3
35	0	0/3	0	1/3

[a] Number of bacteria per gram of organ (mean value for rats with positive cultures).
[b] Number of rats with positive bacterial culture/number studied.
[c] ND, not done.

samples taken from days 3 to 21 after the bacterial injection. The later the samples were taken, the fewer contained positively stained cells. However, in some samples positivity was observed as late as 28 and 35 days after injection. There was no difference in staining pattern between rats injected with 8081 and those given YeO8-116.

The ankle joints of the injected rats were also cultured for the presence of bacteria. When whole ankles with only the skin removed were cultured for yersiniae, both strains were recovered from almost all of the samples taken 3 and 7 days after the bacterial inoculation. At later time points, with one exception, all cultures were negative (Table 4).

TABLE 4. Detection of yersiniae in blood, joints, and synovia

Strain (no. of bacteria injected), postinfection day of sacrificed	Yersinia detection in:			
	Blood (direct, enriched cultures)	Joint (enriched culture)	Synovia (direct, enriched culture)	Synovia (PCR)
YeO8-116 (4×10^7)				
3	0/5,[a] 0/5	3/3	0/2, 1/3	0/2[b]
7	0/5, 0/5	3/3	1/2, 1/2	0/2
14	0/5, 0/5	0/2	0/2, 0/5	0/2
21	0/3, 0/3	ND	ND, 0/3	0/3
28	0/3, 0/3	ND	ND, 0/3	0/2
35	0/3, 0/3	ND	ND, 0/3	0/2
8081 (5×10^7)				
3	0/5, 0/5	3/4	0/2, 1/3	0/2
7	0/5, 0/5	2/5	0/2, 0/2	0/2
14	0/5, 0/5	0/2	0/2, 1/5	0/2
21	0/3, 0/3	ND	ND, 0/3	0/2
28	0/3, 0/3	ND	ND, 0/3	0/2
35	0/3, 0/3	ND	ND, 0/3	0/3

[a] Number of rats positive by bacterial culture/number studied.
[b] Number of synovial specimens with bacterial DNA/number studied.

The samples with whole joints also included the surrounding tissue. Therefore, isolated synovial samples without the surrounding tissue were also cultured. Of the 38 synovial samples taken, 4 were positive, representing both strains equally (Table 4). One sample was positive by direct culture, and the others were positive after enrichment. These results suggested that the numbers of bacteria present in the samples containing isolated synovia were very small and that the bacteria isolated from whole ankle joints most probably originated from the surrounding tissue. Synovial samples taken at each time point were also analyzed by PCR for the presence of bacterial DNA. All of the samples analyzed were negative for bacterial DNA but positive for rat DNA (Table 4). Blood cultures of all of the rats studied were negative (Table 4).

Serology. Development of antibodies specific for YeO:8 was studied in serum samples taken at different time points after bacterial inoculation. Among the strain 8081-injected rats, antibody levels tended to be slightly higher at the time when the arthritis incidence was highest (Fig. 1). However, the only statistically significant difference was in IgM class antibodies on day 35 ($P = 0.048$, Student's t test; Fig. 1). Also, the rats injected with strain YeO8-116/pMW10 or 8081-yoph showed good antibody responses; only IgG responses were lower than in the strain 8081-injected rats ($P < 0.01$ and $P < 0.05$, respectively). As a further control, the same antibody assays were carried out with sera from rats injected with strain 8081-c or 1881 (serotype O:3) or NaCl. Little, if any, antibody activity was observed in these samples (Fig. 1). When the rats developing and not developing arthritis after strain 8081 inoculation were compared for the *Yersinia*-specific antibody response, the arthritic rats at the beginning showed a tendency toward a stronger response (Fig. 2). However, all differences proved to be statistically insignificant ($P > 0.05$).

DISCUSSION

YeO:8 strain 8081 is able to induce experimental arthritis in rats about 2 weeks after intravenous injection of sublethal doses (12, 13, 15, 23, 47). In this work, we demonstrated that YeO8-116, a mutated YeO:8 strain not expressing the *yadA* gene, has a greatly reduced arthritogenic capacity. This was

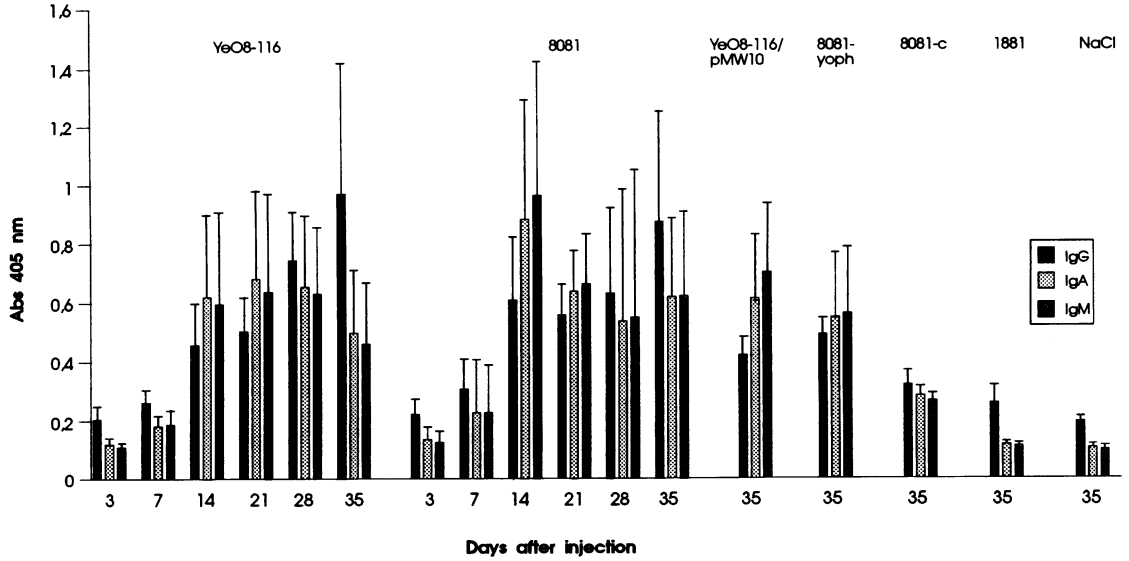

FIG. 1. Antibodies specific for YeO:8 in sera taken at different time points after inoculation of rats with strain YeO8-116, 8081, YeO8-116/pMW10, 8081-yoph, 8081-c, or 1881 or NaCl. The results shown are means plus standard deviations. On days 7 through 28, the number of animals studied was 9 or 10 per group. On day 35, the number was 17 (YeO8-116 injected), 31 (8081 injected), or 5 to 12 (all other groups). Abs, absorbance.

repeatedly observed in comparative experiments utilizing YeO8-116 and parental strain 8081. Arthritis developed in 2 (6%) of 34 strain YeO8-116-injected rats given similar doses which induced arthritis in 22 (73%) of 30 strain 8081-injected rats when the parental strain was used. With plasmid-cured strain 8081-c, the arthritis incidence was zero, as expected. Transcomplementation of the YadA defect by pMW10 returned both the virulence and the arthritogenicity of YeO8-116 to the wild-type level, indicating that the defect in YeO8-116 is due to lack of YadA.

We also used another attenuated strain as a control, strain 8081-yoph, which expressed YadA but not YopH and was clearly less virulent than 8081. It was able to induce arthritis, even though the arthritis was mild and did not last long. This finding confirmed that YadA has a role in the arthritogenicity of YeO:8. It further suggests that YopH is not involved in arthritogenicity in the same way as YadA. YopH blocks the primary host defense by inhibition of phagocytosis (34, 35). When YadA inhibits polymorphonuclear leukocyte phagocytosis, YopH, in turn, inhibits phagocytosis by macrophages, even in the absence of serum opsonins (3, 8). We prefer not to draw too firm conclusions about the contribution of YopH to arthritis induction. However, it seems that YopH may contribute to the severity rather than to the incidence of arthritis. Because 8081-yoph bacteria are more vulnerable to macrophages, they probably do not survive as long as 8081 or YeO8-116 bacteria and the resulting arthritis remains mild in severity and short in duration.

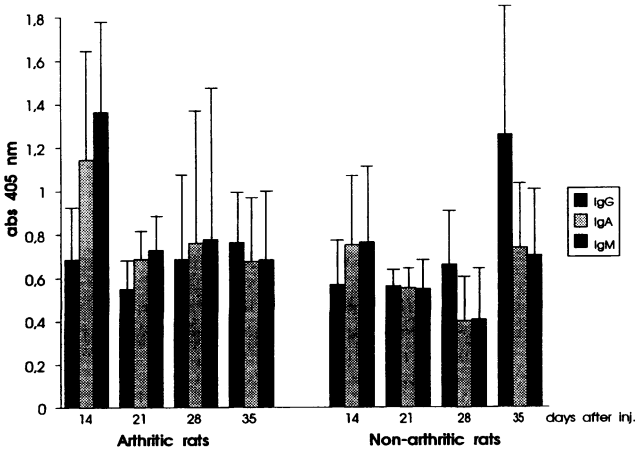

FIG. 2. Antibodies specific for strain 8081 in arthritic and nonarthritic rats. All rats were inoculated with strain 8081. The numbers of animals studied were 3 to 6 on days 14 to 28 and 15 or 16 on day 35. abs, absorbance; inj., injection.

A major question raised by the present results is how much the loss of arthritogenicity in strain YeO8-116 is due to inability to express YadA and to what extent it is a consequence of the slightly reduced virulence. The answer remains open, but the present results, including those obtained with the YopH-negative mutant, leave no doubt about the arthritogenic potential of YadA.

In our studies with the bacteria administered intravenously, 8081 was about six times more virulent than YeO8-116 (Table 2). When administered intraperitoneally or intragastrically to DBA/2 mice, 8081 was 200 or 1,000 times more virulent, respectively, than YeO8-116 (18). Obviously the role of YadA in virulence depends decisively on the route of administration. This is also apparent from the quite similar rates of elimination of the mutant and parental strains after intravenous injections (Table 3). Likewise, both strains are able to induce a vigorous antibody response (Figure 1).

Another important question is whether the bacteria enter the joint tissue or not. A number of the ankle joint cultures at 3 to 7 days after the bacterial inoculation appeared positive following an enrichment period. However, this must be accounted for by the occurrence of bacteria and even microabscesses in the soft tissue surrounding the joints. When plain synovial samples were cultured, considerably fewer samples

were positive. When a number of these samples were tested by PCR with universal bacterial primers with a sensitivity of fewer than 10 bacteria, they appeared negative. In the early phases after bacterial inoculation, live bacteria are present in the synovial samples in minute amounts. Development of human reactive arthritis may also often include a short phase during which live bacteria are present in the joint tissue (48). In any case, the number of bacteria in the rat synovial samples seemed to be too low to be detected by PCR; they were usually detectable by culture only after enrichment. Furthermore, no difference between strains YeO8-116 and 8081 was observed regarding bacterial presence within the joint tissue.

The exact mechanism by which YadA contributes to the arthritogenicity of yersiniae is unknown. Both strains 8081 and YeO8-116 were culturable from the tissues surrounding the joints during the early phases of infection. Thus, binding of YadA to extracellular matrix proteins, including collagen, laminin, and fibronectin (11, 37, 38, 45, 46), may have a role in arthritis development. As a continuation of the present work, we are investigating this possibility by using a YadA mutant defective in collagen binding recently described by us (45).

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