INFECTION AND IMMUNITY, Dec. 1995, p. 4837–4848 Vol. 63, No. 12
0019-9567/95/$04.00+0

Pathogenesis of Defined Invasion Mutants of *Yersinia enterocolitica* in a BALB/c Mouse Model of Infection

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Received 9 June 1995/Returned for modification 1 August 1995/Accepted 11 September 1995

It has been hypothesized for many years that the ability of *Yersinia* spp. to invade tissue culture cells is reflective of their ability to penetrate the intestinal epithelium and that this capacity is an important aspect of the disease process. Three different genes from *Yersinia* spp. that are involved in the tissue culture invasion phenotype have been identified: *inv*, *ail*, and *yadA*. It was previously shown that *inv* is necessary for efficient penetration of the intestinal epithelium by *Yersinia enterocolitica*. The present study was initiated to determine whether other known *Yersinia* invasion factors could promote uptake of the bacteria by mice in the absence of invasin. In addition, the roles of these three invasion factors in the survival of the bacteria, lethality for mice, and development of pathology were compared. We found that YadA is necessary for persistence of *Y. enterocolitica* in Peyer's patches, and consistent with this observation, the *yadA* mutant was avirulent for mice infected either orally or intraperitoneally. In addition, the *inv yadA* double mutant was avirulent. Histological and immunohistological examination of the Peyer's patches of infected mice indicated that despite the presence of large numbers of CFU at 24 h the *yadA* and *ail yadA* mutants cause only minimal pathology and recruitment of macrophages. At 42 h postinfection, Peyer's patches from mice infected with the *inv* mutant showed no pathology, despite the prediction that some of the mice by this time would be colonized. However, at 72 h, inflammation and necrosis were evident in some Peyer's patches. Together, these observations suggest that for visible pathology to develop, a threshold number of bacteria ($>10^5$) is needed and the bacteria need to persist for more than 24 h. Lastly, YadA but not Ail may play a role in the less efficient, delayed invasion of the intestinal epithelium observed for the *inv* mutant.

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are pathogenic for humans, causing syndromes ranging from enteritis to acute mesenteric lymphadenitis (16). Organisms are ingested in contaminated food or water (16, 22), after which they travel to the terminal ileum and bind to the intestinal epithelium (27). The bacteria then penetrate the intestinal mucosa through M cells (25, 27, 29), specialized cells involved in intestinal antigen uptake (56, 57, 86–88). After penetration of the intestinal epithelium, *Y. enterocolitica* colonizes the local lymphoid follicles, known as Peyer's patches, and can eventually spread through the lymphatics to disseminate to the liver and spleen (18). However, disseminated *Y. enterocolitica* infections of humans are rare and occur primarily in immunocompromised individuals. The ability to survive within these tissues is associated with the presence of a 70-kb virulence plasmid (pYV) that is essential for the pathogenesis of *Yersinia* spp. (21, 31); however, strains cured of pYV are still able to cross the intestinal epithelium in an animal model (8, 29, 43, 68).

It has been established that the invasive ability of enteropathogenic *Yersinia* spp. correlates with their ability to cause disease (42, 52, 60, 73, 81). Molecular analysis of enteropathogenic invasion by *Yersinia* spp. has led to the identification of three unique invasion genes (*inv*, *ail*, and *yadA*) that are able to independently promote invasion (34, 51, 89). The chromosomal *inv* gene product, invasin, is the principal invasion factor for both *Y. enterocolitica* and *Y. pseudotuberculosis* (36, 63). Invasin binds directly to a subset of β_1-chain integrin receptors with high affinity, which potentiates the ensuing internalization of bacteria (35, 79).

A second *Y. enterocolitica* chromosomal invasion gene, *ail*, was cloned simultaneously with the *inv* gene, but unlike that with invasin, Ail-mediated internalization shows cell line specificity (51). A *Y. enterocolitica ail* mutant is reduced for entry into tissue culture cells (64), but the reduction is less than that observed for an *inv* mutant (63). The tissue culture invasion phenotype of an *inv* mutant is sharply reduced, but a residual level of uptake is detectable when *Yersinia* spp. harbor the pYV plasmid (33, 63). This low-level, plasmid-dependent entry was recently shown to be mediated by YadA in *Y. pseudotuberculosis*, and like invasin, YadA may also utilize β_1-chain integrin receptors for internalization (12, 89). The role of YadA in tissue culture invasion by *Y. enterocolitica* has not been examined.

In a mouse infection model, a *Y. enterocolitica inv* mutant is severely impaired in its ability to cross the intestinal epithelium and colonized Peyer's patches early after infection, which parallels tissue culture invasion assay results (63). Although invasin appears to be the primary factor required for penetration of the intestinal epithelium, some *inv* mutants eventually breach this barrier. At 42 h postinfection, the Peyer's patches of approximately half of the mice become colonized at nearly wild-type levels (63). While factors promoting this delayed invasion phenotype have not yet been identified, Ail and YadA are

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Description	Source or reference
Y. enterocolitica		
8081v	*ail*$^+$ *yadA*$^+$ *inv*$^+$	65
8081(pYV085)	*ail*$^+$ *yadA*$^+$ *inv*$^+$	This study
YE2v	*ail yadA*$^+$ *inv*$^+$	85
YE3c	*ail*$^+$/*ail yadA inv*$^+$	85
8081(pYV08-116)	*ail*$^+$ *yadA inv*$^+$	This study
8081(pYV08-116, pMW10)	*ail*$^+$ *yadA*$^+$ *inv*$^+$	This study
8081(pYV08-11, pCJ179)	*ail*$^+$ *yadA inv*$^+$	This study
JP273v	*ail*$^+$ *yadA*$^+$ *inv*	63
YE2(pYV08-116)	*ail yadA inv*$^+$	This study
YE3(pYV08-116)	*ail*$^+$/*ail yadA inv*$^+$	This study
JP275v	*ail yadA*$^+$ *inv*	This study
JP273(pYV08-116)	*ail*$^+$ *yadA inv*	This study
JP275(pYV08-116)	*ail yadA inv*	This study
Plasmids		
pYV08	Wild-type virulence plasmid	65
pYV085	pYV08 with *kan* just downstream of *yadA*	78
pYV08-116	pYV08 with *yadA*::*kan*	26
pMW10	pCJ179 with *yadA*	This study
pVM118	*ail* suicide vector	85
pCJ179	Low-copy vector	This study

[a] All *Y. enterocolitica* strains used in this study are nalidixic acid resistant, and all *ail* mutant and merodiploid strains contain an uncharacterized chromosomal rearrangement (see reference 85). All *ail* mutants were constructed with the use of pVM118; all *yadA* mutants were constructed with the use of pYV08-116 (a gift of M. Skurnik). All strains with the designation "v" contain the wild-type virulence plasmid, pYV08; strains with the designation "c" are cured of the virulence plasmid.

likely candidates. Evidence supporting this hypothesis is that *yadA* is expressed in the intestinal lumen within 1 h of infection of rats (77) and mediates adherence to rabbit intestinal tissue (58). Likewise, *ail* is expressed in mouse Peyer's patches at 48 h postinfection (85). To address whether or not these genes are involved in *Y. enterocolitica* invasion, quantitative assessments of intestinal epithelial invasion and survival within Peyer's patches were made with the following isogenic mutants: *inv*, *ail*, *yadA*, *inv ail*, *inv yadA*, *ail yadA*, and *inv ail yadA*. We further report histological and immunohistological analyses of infected mice that describe the pathology caused by wild-type *Y. enterocolitica* and the invasion mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are described in Table 1. Strains were stored at −80°C in L broth (Difco Laboratories, Detroit, Mich.) containing 25% (vol/vol) glycerol. *Y. enterocolitica* was grown in L broth, on Luria-Bertani agar plates, or on M9 minimal agar plates supplemented with 0.2% glucose and 0.0001% vitamin B_1 at 23 or 37°C with aeration (49). Antibiotics were used at the following concentrations when appropriate: chloramphenicol, 20 μg/ml; kanamycin, 40 μg/ml; nalidixic acid, 30 μg/ml; and tetracycline, 10 μg/ml. The different pYV derivatives were introduced into *Y. enterocolitica* by electroporation with a Gene Pulser (Bio-Rad) (23, 38); concentrated bacteria prepared according to the manufacturer's recommendations were mixed with 2 μg of DNA and subjected to one pulse at 400 Ω, 850 V, and 25 μF. *Y. enterocolitica* strains were cured of the virulence plasmid by streaking them on Luria-Bertani agar plates containing 20 mM $MgCl_2$ and 20 mM NaC_2O_4 and incubating the plates overnight at 37°C (11). Large, plasmid-cured colonies were isolated and verified for plasmid loss by examination of plasmid DNA.

Nucleic acid purification and probe preparation. Plasmid DNA was isolated by the alkaline lysis method (45) or with Magic/Wizard Minipreps (Promega, Madison, Wis.). DNA fragments used in plasmid construction were prepared by digestion with the appropriate restriction endonucleases and then by gel purification with Gene Clean (Bio 101, La Jolla, Calif.). DNA restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and Klenow fragment were purchased from New England Biolabs (Beverly, Mass.) and used according to the instructions of the manufacturer.

Tissue culture invasion assay. HEp-2 and CHO cells were maintained in RPMI 1640 medium (20 mM L-glutamine) containing 5 and 10% fetal bovine serum, respectively. Bacterial internalization was determined by the gentamicin protection assay as described previously (51); bacteria were incubated with the monolayer for 90 min prior to being washed with phosphate-buffered saline (PBS) and treated with gentamicin. The efficiency of internalization was defined as the number of CFU that survived gentamicin treatment relative to the number in the initial bacterial inoculum.

Restoration of wild-type *yadA* to the *yadA* mutant: construction of 8081 (pYV08-116, pMW10). The low-copy, mobilizable *yadA* complementing plasmid was constructed as follows. A 600-bp *Nde*I-*Hin*dIII fragment was dropped out of pCP178 (60a) and end filled, and a *Bgl*II linker was inserted to generate pCJ178, a low-copy (pSC101-derivative) plasmid that encodes chloramphenicol acetyltransferase. The 1,800-bp *Bam*HI fragment containing *mob*RP4 derived from pJM703.1 (53) was subcloned into the *Bgl*II site of pCJ178 to generate pCJ179. Plasmid pRM101.14 (a gift from R. J. Martinez, University of California at Los Angeles) was digested with *Eco*RI to release a 5,200-bp fragment containing *yadA*, which was subcloned into pCJ179 to generate pMW10. No known genes or open reading frames other than *yadA* have been identified within this *yadA*-containing DNA fragment.

Filter matings (75) of pMW10 were conducted with *yadA* mutant strain 8081 (pYV08-116) by using the transfer functions supplied from plasmid pRK2013 (24). The *Yersinia* strain was heated prior to mating to reduce host restriction of incoming DNA (20). The resulting chloramphenicol-resistant exconjugants were verified for the presence of pMW10 by analyzing purified plasmid DNA from the exconjugates. *Yersinia* strains that were shown to contain pMW10 were also verified for *yadA* expression from pMW10 (overnight growth in Luria-Bertani broth at 37°C) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (40) of total cellular proteins (10) (data not shown).

Restoration of wild-type *ail* to the *ail yadA* mutant: construction of YE3(pYV08-116). The construction of the *ail* merodiploid strain, YE3v, has been described by Wachtel and Miller (85). Briefly, suicide plasmid pVM118 containing *ail*::miniTn*10* was mated into 8081v^{Nal} as described above. A single crossover event resulted in merodiploid YE3v, and production of Ail by this strain was verified by Western blot (immunoblot) analysis (85). This strain was cured of the virulence plasmid as described above and is referred to as YE3c. The *yadA* mutation was introduced into YE3c by electroporation of pYV08-116 as described above.

Mouse assays. *Y. enterocolitica* was grown with aeration for 18 h at 23°C, washed with PBS, and diluted to the appropriate infectious dose. Virus-free, 6- to 7-week-old female BALB/c mice were infected either intragastrically (i.g.) or intraperitoneally (i.p.). The in vivo invasion and 50% lethal dose (LD_{50}) assays were performed as described previously (63). Each experiment was conducted at least twice. Animal protocols were approved by the University of California at Los Angeles Animal Research Committee.

Histological studies and immunohistochemistry. For histological studies, mice were infected with 5×10^8 bacteria and sacrificed at 42 h postinfection. Since *Y. enterocolitica* is thought to invade the Peyer's patches of the terminal ileum, the Peyer's patches harvested for paraffin sectioning were alternated with those intended for frozen sectioning in some experiments. This enabled us to select samples for immunohistochemical studies that were representative of the pathology displayed by each strain. For these experiments, six to eight Peyer's patches proximal to the ileal-cecal junction were harvested; alternating Peyer's patches were harvested for some strains (wild type and *ail*, *yadA*, *ail yadA*, and *inv* mutants), while in other cases, four Peyer's patches were harvested from individual mice and used for either frozen or paraffin sectioning (*inv ail*, *inv yadA*, and *inv ail yadA* mutants).

Peyer's patches intended for paraffin sectioning were placed in HistoPrep OmniSette tissue cassettes (Fisher Scientific, Pittsburgh, Pa.), fixed in 3.7% formaldehyde for a minimum of 48 h, and processed for paraffin embedding (66). The samples were embedded in paraffin, and at least two 4-μm-thick step cuts were sectioned with a Jung Biocut 2035 Microtome (Leica, Wetzlar, Germany). Samples were stained with hematoxylin (5%, wt/vol) and eosin (1%, wt/vol) (Fisher Chemicals, Fair Lawn, N.J.) (66). Samples were prepared for Gram staining and Steiner-Steiner staining according to the methods of Prophet et al. (66) and Sheehan and Hrapchak (74).

Peyer's patches intended for frozen sectioning were placed in Tissue-Tek II Cryomolds (Miles Laboratories, Naperville, Ill.) and quick-frozen in a dry ice-isopentane (2-methyl butane; Mallinckrodt Specialty Chemicals Co., Paris, Ky.) bath for 1 min in Tissue-Tek O.C.T. Compound (Miles Laboratories Diagnostic Division, Elkhart, Ind.) (10.24% [wt/wt] polyvinyl alcohol and 4.26% [wt/wt] polyethylene glycerol). The samples were wrapped in parafilm M (American Can Co., Greenwich, Conn.) and stored at −80°C prior to sectioning. Frozen sections (4 to 6 μm thick) were cut with a Tissue Tech Cryostat (Miles Laboratories) and placed on superfrost plus slides (Fisher Chemicals). The sections were fixed in 10% neutral buffered saline formalin for 14 min at 4°C and rehydrated in PBS (pH 7.4) for 5 min. Blocking was carried out with 3% hydrogen peroxide in water for 5 min, which was followed by a rinse in tap water and incubation in PBS for

TABLE 2. Tissue culture invasion phenotype and virulence of *Y. enterocolitica* invasion mutants

Strain	Invasion factor(s)	Invasion (%)[a]		LD_{50} (no. of organisms)[b]	
		HEp-2	CHO	i.g.	i.p.
8081c	Inv^+ and Ail^+	56.2 ± 10.0	86.3 ± 0.8	ND	ND
8081v	Inv^+ Ail^+, and $YadA^+$	13.1 ± 1.0	28.8 ± 1.2	$10^{5.8}$	$10^{3.2}$
8081(pYV08-116)	Inv^+ and Ail^+	12.6 ± 1.3	25.0 ± 2.1	$>10^{10.4}$	$10^{7.0}$
YE2c	Inv^+	ND	71.7 ± 7.8	ND	ND
YE2v	Inv^+ and $YadA^+$	11.8 ± 1.1	24.2 ± 2.6	$10^{5.7}$	ND
YE2(pYV08-116)	Inv^+	ND	20.1 ± 1.0	$>10^{10.3}$	ND
JP273c	Ail^+	0.034 ± 0.01	0.365 ± 0.01	ND	ND
JP273v	Ail^+ and $YadA^+$	0.358 ± 0.002	1.10 ± 0.08	$10^{6.0}$	$10^{3.4}$
JP273(pYV08-116)	Ail^+	0.044 ± 0.01	0.391 ± 0.02	$>10^{10.3}$	$10^{7.0}$
JP275c		0.027 ± 0.001	0.318 ± 0.02	ND	ND
JP275v	$YadA^+$	0.323 ± 0.01	0.989 ± 0.04	$10^{6.5}$	$10^{3.3}$
JP275(pYV08-116)		0.024 ± 0.002	0.280 ± 0.001	$>10^{10.3}$	ND

[a] For invasion assays, fresh overnight cultures grown at 23°C were used to seed subconfluent monolayers at a multiplicity of infection of ~100. The percent invasion is the percent bacteria that survive gentamicin treatment relative to the initial inoculum. Values are averages of duplicate samples ± the range and reflect results from several experiments. ND, not determined.

[b] LD_{50}s were determined according to the method of Reed and Meunch (67). Experiments were done a minimum of two times. ND, not determined.

5 min. The subsequent steps were carried out in a humid chamber. The samples were blocked in 10% normal goat serum (Dako Corp., Carpinteria, Calif.) for 10 min at room temperature, and the primary antibody was applied and allowed to incubate overnight at 4°C. The primary antibodies used were a rabbit anti-invasin polyclonal antibody (61) and a rabbit anti-mouse macrophage polyclonal antibody preabsorbed to mouse erythrocytes (Inter-Cell Technologies, Inc., Hopewell, N.J.). The anti-invasin antibody was diluted 1:500 in 2% bovine serum albumin (BSA) in PBS, and the anti-mouse macrophage antibody was diluted 1:50,000 in 2% BSA in PBS. The anti-invasin antibody is capable of recognizing truncated forms of invasin, such as those produced by the *inv* mutant JP273v. The anti-mouse macrophage antibody used was a polyclonal anti-macrophage antibody raised against mouse peritoneal macrophages, and it identifies both mature and immature mouse macrophages. The primary antibody was washed off three times in PBS for a total of 5 min, after which the secondary antibody, diluted 1:50 in 2% BSA in PBS, was applied for 30 min at room temperature. The secondary antibody used was a goat anti-rabbit immunoglobulin G heavy-plus-light-chain antibody conjugated to horseradish peroxidase (Zymed Laboratories, Inc., South San Francisco, Calif.). Following a rinsing step (three times in PBS for a total of 5 min), the samples were developed in a 3-amino-9-ethyl carbozyl–dimethyl sulfoxide mixture (Sigma Chemical Co., St. Louis, Mo.) for 10 min. Control sections were also cut for each tissue block, exposed to either no primary antibody or secondary antibody or to primary antibody with no secondary antibody, and similarly developed to determine the degree of background staining. Representative samples for each invasion mutant were chosen for immunohistochemical analysis at random (72-h study) or on the basis of hematoxylin-eosin pathology (42-h study).

RESULTS

Invasion phenotypes of different *Y. enterocolitica* invasion mutants in the tissue culture invasion assay. It has been well documented that by itself, invasin is an efficient adhesin and invasion factor (36, 62, 90), and Ail has been shown to play a minor but detectable role in the invasion phenotype of *Y. enterocolitica* (64, 85). Recently, it was demonstrated that YadA contributes to the invasion phenotype of *Y. pseudotuberculosis*; however, the role of YadA as an invasion factor for *Y. enterocolitica* has not been defined. To compare the contributions of *yadA* to the invasion phenotype of *Y. enterocolitica* 8081 for both HEp-2 and CHO cells, the entry efficiency of the wild type was compared with those of different isogenic invasion mutants. The *Y. enterocolitica yadA* gene was disrupted with a kanamycin resistance cassette and crossed onto plasmid pYV08 to generate plasmid pYV08-116 (26). Plasmid pYV085 (78) has the kanamycin resistance cassette inserted downstream of *yadA*, and this plasmid served as a control of nonspecific effects resulting from the presence of the resistance gene.

The *yadA* mutant entered cultured cells as efficiently as parental strain 8081 (Table 2). The strains harboring pYV08 or pYV08-116 showed invasion levels lower than those of the cured strains, which was most likely due to the pYV-encoded cytotoxic factors, YopE and YopH (12, 70, 71). Regardless, invasin is such a dominant invasion factor that it might be masking the effect of the *yadA* mutation. The *Y. enterocolitica inv* mutant JP273 was used to examine the YadA pathway of cellular entry to determine whether or not this was masked by invasin-promoted internalization. JP273 exhibited a 30- to 1,600-fold decrease in invasion of cultured cells compared with the wild type (Table 2) (63). However, JP273v was consistently up to 10-fold more invasive than JP273c. This increased internalization appeared to be associated with the presence of pYV08, and it seemed likely that it was mediated by YadA, as was recently shown for *Y. pseudotuberculosis* (12, 89).

This was investigated further by examining the internalization efficiency of the *Y. enterocolitica inv yadA* double mutant, JP273(pYV08-116). The internalization of JP273(pYV08-116) was similar to that of JP273c and was reduced relative to that of JP273v (Table 2); thus, *yadA* appeared to mediate low-level internalization that was detectable in an *inv* background. The results described above are consistent with the notion that the pYV-encoded invasion factor for *Y. enterocolitica* was YadA.

The *ail* gene is maximally expressed at 37°C (50, 64); therefore, the contribution of Ail to the invasive phenotype of *Y. enterocolitica* in mutant JP273c or JP273(pYV08-116) was not apparent in the cultures grown at 23°C (Table 2). When they were grown at 37°C, these strains did show a three- to fourfold increase in invasion of CHO cells only, which was abolished in an *inv ail yadA* triple mutant [JP275(pYV08-116)] or an *inv ail* mutant cured of pYV08 (JP275c) (data not shown). The invasion phenotypes of JP275v, JP275c, and JP275(pYV08-116) grown at 23°C are essentially identical to those of the JP273 strains that have an intact *ail* gene (Table 2). However, Ail does promote low-level entry of *Y. enterocolitica* grown at 37°C (64, 85).

In vivo invasion phenotypes of different *Y. enterocolitica* invasion mutants. The number of CFU recovered from Peyer's patches very early after the mice were i.g. infected reflects the initial invasion of the intestinal mucosa. Invasin was shown to be of primary importance for *Y. enterocolitica* in this in vivo invasion assay (63). Despite lacking invasin, the *inv* mutant eventually did reach deeper tissues in a few mice, which indicated that alternative bacterial invasion factors may have mediated this delayed entry. Although Ail and YadA appear to

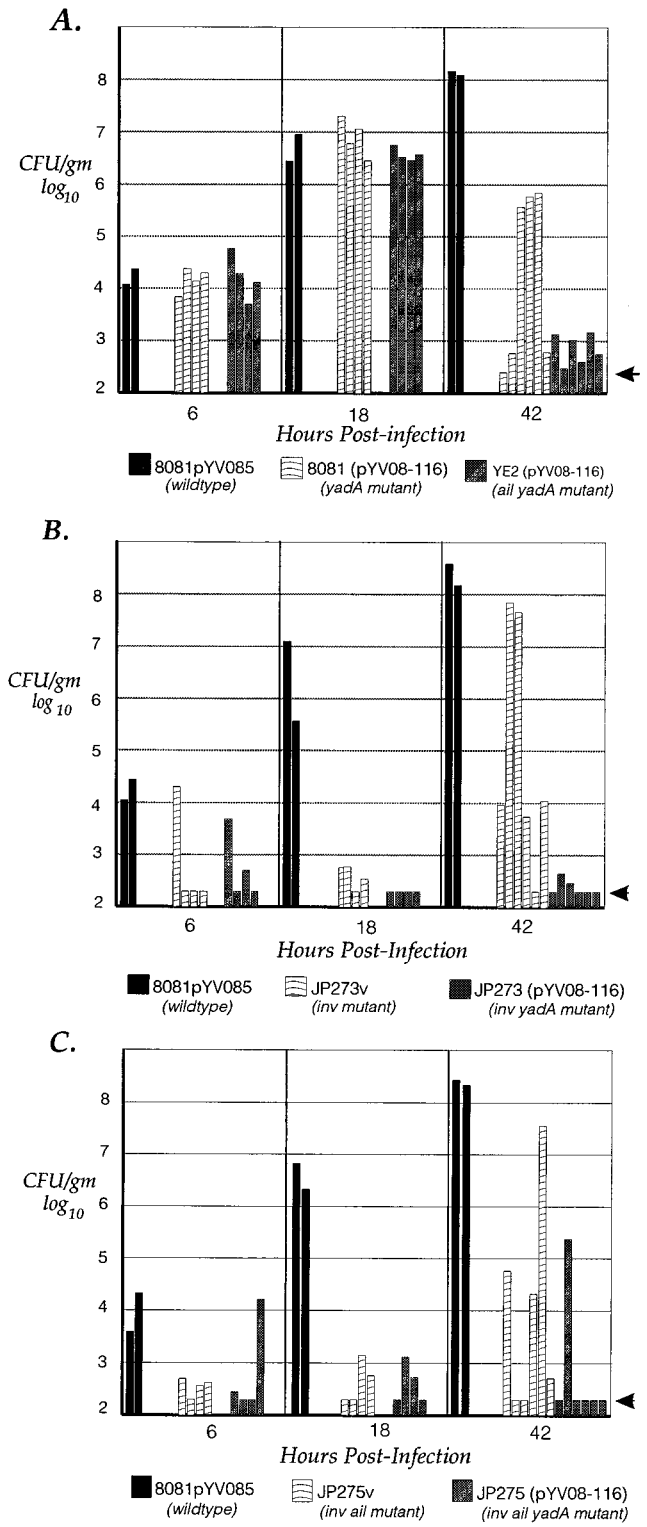

FIG. 1. Colonization of murine Peyer's patches by different *Y. enterocolitica* invasion mutants. BALB/c mice were infected with ~10^8 bacteria, and their Peyer's patches were harvested at 6, 18, and 42 h postinfection. Each bar represents the number of CFU recovered from a single mouse. The arrows on the right indicate the limit of detection for this assay (300 CFU/g of Peyer's patch).

make only a minor contribution to *Y. enterocolitica* invasion of tissue culture cells, their roles may be more significant in vivo.

Each of the mutants was individually tested for in vivo invasion by infecting mice i.g. and quantitating the CFU recovered from Peyer's patches at 6, 18, and 42 h postinfection. *Y. enterocolitica* 8081(pYV085) (wild type) showed a gradual increase in Peyer's patch colonization, with uniformly high bacterial numbers at 42 h (Fig. 1A); this is comparable to what was previously observed for 8081v and indicates that the presence of the kanamycin resistance cassette on pYV08 does not affect Peyer's patch colonization. The *ail* mutant (YE2v) showed similar results (85). At 6 and 18 h, the *yadA* and *ail yadA* mutants invaded and colonized Peyer's patches as well as the wild type did (Fig. 1A). At 42 h, only the *ail* mutant behaved like the wild type (85), while Peyer's patch colonization by the *yadA* mutant was reduced an average 3 orders of magnitude compared with that of the wild type (Fig. 1A). Moreover, colonization by the *ail yadA* double mutant was decreased even further; approximately 10^5 fewer CFU was recovered from the Peyer's patches of mice infected with this mutant than was recovered from the wild type. These results suggest that YadA and possibly Ail contribute to the persistence of *Y. enterocolitica* in Peyer's patches.

All of the strains that contain an *inv* mutation were defective in their ability to invade and colonize Peyer's patches at 6 and 18 h (Fig. 1B and C). At 42 h, mice infected with the *inv ail* (JP275v) double mutant fell into two groups, those with highly colonized and those with poorly or noncolonized Peyer's patches, as was previously observed for the single *inv* mutant JP273v (Fig. 1B) (63). Conversely, the *inv yadA* double mutant [JP273(pYV08-116)] and the *inv ail yadA* triple mutant [JP275(pYV08-116)] were never able to consistently invade or colonize the Peyer's patches (Fig. 1B and C). These data reconfirm that invasin is necessary for efficient penetration of the intestinal epithelium but is not needed for survival (63). These results also suggest that *yadA* contributes to the delayed invasion of Peyer's patches observed for the *inv* mutant. However, a *yadA* mutation in any genetic background of *Y. enterocolitica* affected the persistence of this organism in Peyer's patches (Fig. 1 [42 h]); thus, because of the effect of the *yadA* mutation on persistence, it is difficult to definitively conclude that YadA contributes to the eventual penetration of Peyer's patches by the *inv* mutant.

To verify that the phenotype observed for the *yadA* mutant was the result of the loss of YadA and not an artifact of strain construction, this mutation was complemented in *trans* and tested for its ability to survive and proliferate in murine Peyer's patches. The low-copy, *yadA*-complementing plasmid, pMW10, was constructed and conjugated into the *yadA* mutant and into the wild-type strain. The resulting strains along with the *yadA* mutant carrying cloning vector pCJ179 were tested for survival in Peyer's patches. Plasmid pMW10 was able to complement the *yadA* mutant and promote its survival in Peyer's patches. In contrast, the *yadA* mutant carrying cloning vector pCJ179 did not survive well in Peyer's patches. The wild type alone as well as the wild type carrying pMW10 survived in Peyer's patches equally well (data not shown). Furthermore, the kanamycin resistance cassette insert in pYV085 (just downstream of the *yadA* coding sequence) had no effect on the survival of 8081(pYV085) (Fig. 1), further suggesting that the insert in pYV08-116 is unlikely to have a polar effect downstream of *yadA*.

The *Y. enterocolitica ail yadA* mutant [YE2(pYV08-116)] was more severely affected than the *yadA* mutant for persistence in the Peyer's patches at 42 h postinfection (Fig. 1A). To determine if Ail rather than the chromosomal rearrangement

found in association with the *ail* mutation (85) was responsible for this phenotype, complementation experiments were performed. *ail* was initially provided in *trans* on a low-copy plasmid in YE2(pYV08-116). However, this plasmid was extremely unstable in vivo, even in a wild-type-strain background. The plasmid instability persisted, despite attempts to inject mice subcutaneously with antibiotics, the resistances of which were encoded by the plasmid (data not shown). *ail* is known to be maximally expressed at 37°C and is believed to have a very strong promoter (64, 85a). It is possible that increased expression of *ail* is detrimental, even when it is carried on a low-copy plasmid, and thus is selected against in vivo.

Because it was not possible to complement *ail* in *trans* on a low-copy plasmid, the *ail* merodiploid strain, YE3c, was utilized for in vivo complementation experiments. This strain contains the same chromosomal rearrangement as that found in the original *ail* mutant, YE2v, and is cured of the virulence plasmid. The pYV plasmid carrying the *yadA* mutation was transformed into YE3c, creating YE3(pYV08-116). This strain was phenotypically similar to *ail yadA* mutant YE2(pYV08-116) and failed to persist in mouse Peyer's patches at 42 h postinfection (data not shown). YE3(pYV08-116) was recovered from Peyer's patches and grown in vitro, and whole-cell lysates were collected from the growing culture. Western blots indicated that this strain produced Ail in vitro (data not shown). YE3(pYV08-116) had a growth rate identical to those of wild-type strain 8081v as well as *ail yadA* mutant YE2(pYV08-116) (data not shown). These results indicate that decreased persistence in the Peyer's patches by the *ail yadA* double mutant relative to that of the *yadA* single mutant may be due to the chromosomal rearrangement rather than to the loss of Ail.

LD_{50}s (i.g. and i.p.) of different *Y. enterocolitica* invasion mutants. The course of a *Y. enterocolitica inv* mutant infection is altered by comparison with that of the wild type, but the virulence of this strain remains unaffected, as measured by the LD_{50} (63). The course of a *Y. enterocolitica yadA* mutant infection in i.g. infected mice was already altered within 2 days; therefore, LD_{50}s were determined so that the effect of *yadA* on the systemic phase of infection could be evaluated.

The *inv*, *ail*, and *inv ail* mutants were as virulent as the wild type, regardless of whether the route of infection was i.g. or i.p. (Table 2). One observable difference with i.g. infections was that the average time to death for mice infected with the *inv ail* double mutant (JP275v) was delayed by one day; on average, mice infected i.g. with the wild type died on day 7.5, whereas mice infected with the *inv ail* double mutant died on day 8.5. In contrast, all strains with a *yadA* mutation were completely avirulent (Table 2). The i.g. LD_{50}s were not measurable, because the values were greater than 4 orders of magnitude higher than that for the wild type (i.e., less virulent). These results also indicated, as did the in vivo invasion results, that *Y. enterocolitica* cannot survive host defenses in the absence of YadA. Whether this is due to an increased sensitivity to host bactericidal activity or to an increased rate of clearance is not known at this time. Furthermore, the inability of the *Y. enterocolitica yadA* mutant to survive in Peyer's patches was not restricted to this tissue, because the *yadA* mutant was avirulent even when introduced by the i.p. route (Table 2).

Histological examination of Peyer's patches from mice infected with the wild type and invasion and survival mutants of *Y. enterocolitica*. To further investigate the effect of *ail*, *yadA*, and *inv* mutations on the interactions of *Y. enterocolitica* with the host, the Peyer's patches of mice infected with various mutants were examined for pathology visible at 42 h postinfection. Alternating Peyer's patches were harvested from the terminal ileum, and they were sectioned, stained with hematoxylin-eosin, and examined microscopically. Alternate Peyer's patches were examined by immunohistochemical analysis as described below. Peyer's patches were characterized as displaying both inflammation and necrosis, inflammation only, or no observable pathology. These and subsequent specimens were analyzed by a pathologist who had no knowledge of the grouping or identity of the tissue specimens.

Peyer's patches with both inflammation and necrosis contained microabscesses within the lymphoid parenchyma (Fig. 2A and B). The microabscesses had poorly defined margins, with a predominant recruitment of polymorphonuclear leukocytes (PMNs). However, an underlying network of large mononuclear cells was also present. Cell death and necrotic tissue were apparent at the center of each focus (Fig. 2A and B [arrowheads]). The general region of the microabscesses was essentially cleared of the lymphocytic mix characteristic of mock-infected control Peyer's patches. The ileal epithelium adjacent to the Peyer's patches was surprisingly intact, without evidence of inflammatory infiltrates.

Peyer's patches with inflammation only had an appearance similar to those with inflammation and necrosis, with recruitment of numerous PMNs, an underlying network of large mononuclear cells, and clearing of the lymphocytic aggregates characteristic of uninfected controls (Fig. 2C and D). Again, the margins of affected areas were poorly defined. No necrosis was noted centrally, and no granulomatous change was present. As was noted for the microabscesses with necrosis, normal bowel epithelial architecture was present. This general finding in all affected foci suggests that disruption of the epithelium plays no role in the invasion by *Y. enterocolitica* at this time point.

Mice infected with the wild type or the *ail* mutant contained the largest number of Peyer's patches exhibiting both inflammation and necrosis, in agreement with the large number of bacteria detected in the Peyer's patches at 42 h postinfection (Fig. 3). Interestingly, the *yadA* mutant had pathology similar to that of the *ail yadA* mutant, with a few inflamed Peyer's patches and no visible necrosis (Fig. 3). This was seen despite a 2.5-log difference in the number of recoverable bacteria from these mutants at 42 h postinfection. Another interesting feature was the complete lack of pathology in the Peyer's patches of mice infected with the *inv* mutant (Fig. 3A). Previous studies of Peyer's patch invasion by the *inv* mutant at 32 h postinfection revealed two distinct populations of mice; some mice do not have detectable bacteria in the Peyer's patches, whereas others have high numbers of bacteria (63). However, two of the four mice infected with the *inv ail* mutant had one necrotic Peyer's patch. Pathology conferred by the *inv* mutant was also examined at 72 h postinfection to determine if pathology was detectable at a later time point in the subpopulation of mice that become infected with the *inv* mutant. At 72 h postinfection, at least one of four Peyer's patches from each of the infected mice displayed both inflammation and necrosis, showing that severe inflammatory changes can occur in mice infected with the *inv* mutant, despite the delayed invasion kinetics (Fig. 3B).

Little or no pathology was exhibited in the Peyer's patches of mice infected with the *inv yadA* or *inv ail yadA* mutant. The lack of inflammation and necrosis in Peyer's patches from mice infected with these mutants was expected, given the lack of invasion and survival in the Peyer's patches displayed by these mutants at 42 h postinfection.

To correlate pathology with bacterial localization, selected samples from the study described above were sectioned further and examined after being stained with Gram stain or Steiner-

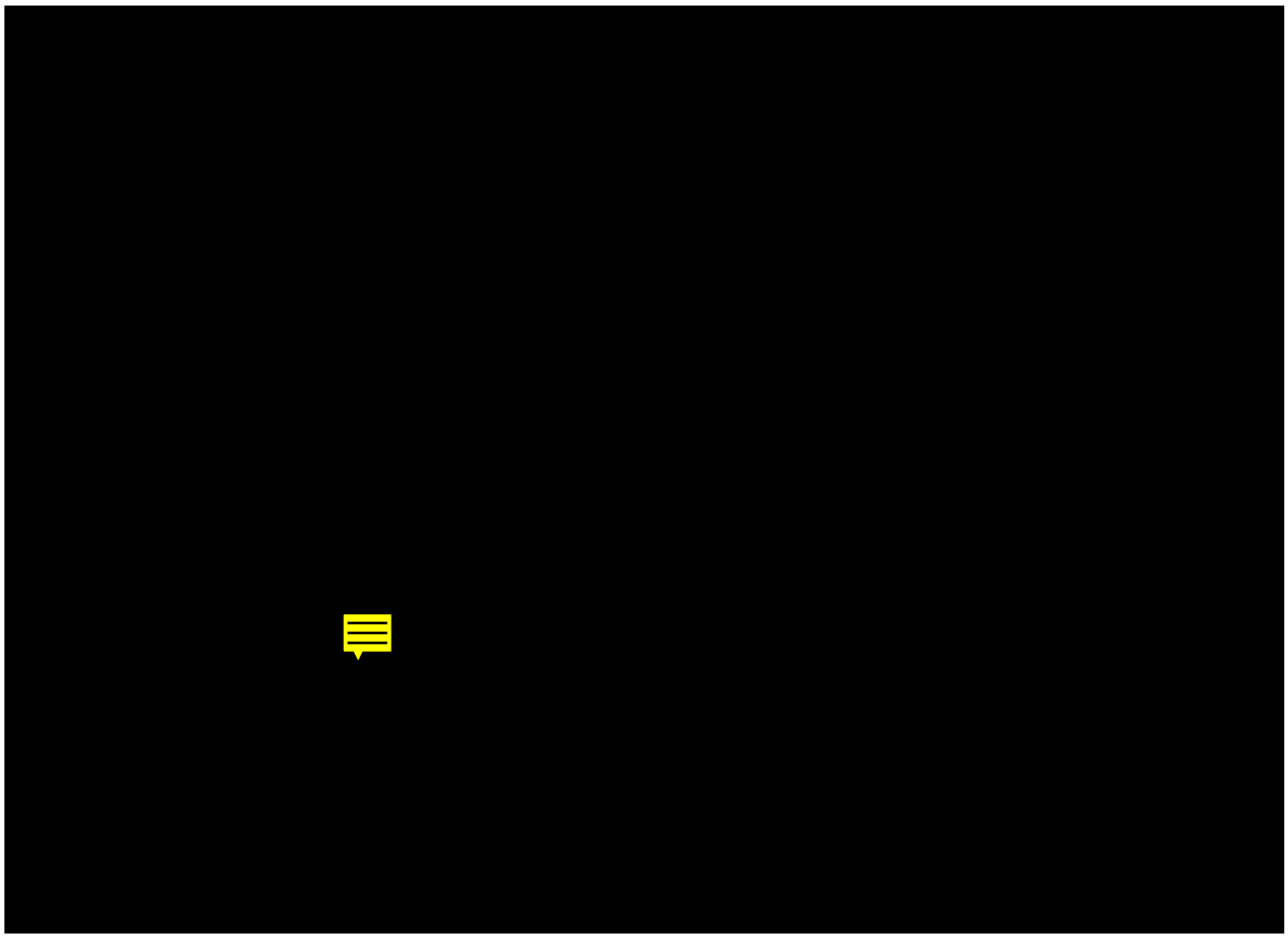

FIG. 2. Histological examination of murine Peyer's patches infected with wild-type *Y. enterocolitica*. BALB/c mice were infected i.g. with approximately 5×10^8 bacteria, and the Peyer's patches were harvested at 42 h postinfection. Tissues were stained with hematoxylin and eosin as described in Materials and Methods. Tissues were scored individually in a blind study for the presence of inflammation and necrosis and inflammation only. (A) Example of inflammation and necrosis. Areas of cell death and necrosis are indicated by arrowheads. Magnification, ×6.5. (B) Example of inflammation and necrosis. The arrowhead shows inflammation and necrosis within a single follicle. Note that not all follicles showed inflammation and necrosis. Magnification, ca. ×1.6. (C) Example of inflammation only. Magnification, ×6.5. (D) Example of mock-infected negative control. Normal bowel epithelium is indicated by the arrowhead. Magnification, ca. ×1.6.

Steiner stain (a silver deposition identification) so that bacterial cells could be visualized (66, 74). Of the samples tested (the wild type and the *ail* and *ail yadA* mutants), the wild type and the *ail* mutant were positive for both stains, displaying gram-negative coccobacillary forms near the center of the necrotic foci. Sections stained positively with at least one stain wherever necrotic foci were seen only with rare exceptions. The *ail yadA* mutant displayed no positive staining in either areas of inflammation or regions that contained no visible pathology (data not shown). The positive bacterial staining localized to the necrotic foci is a further indication that these regions were bacterial overgrowth sites. However, since both of these stains are nonspecific, immunohistochemistry was performed to identify the bacteria as *Y. enterocolitica.*

Immunohistochemical analysis of *Y. enterocolitica* mutants in the Peyer's patches correlates with pathological phenotype. To definitively identify the location of the *Y. enterocolitica* strains in the Peyer's patches, we used immunohistochemical analysis. Sections from frozen tissue were incubated with an anti-invasin antibody as a general *Y. enterocolitica* marker to determine the location of bacteria relative to Peyer's patch pathology. This antibody is capable of recognizing truncated forms of invasin, such as those produced by the *inv* mutant; invasin is known to be expressed in mouse Peyer's patches at 42 h postinfection (61). To investigate the primary immune response of mice to *Y. enterocolitica* infection, which is likely to involve circulating macrophages, the samples were also incubated with an anti-mouse macrophage antibody. Early inflammatory responses consist of morphologically recognizable PMNs and an underlying network of large mononuclear cells. The anti-mouse macrophage antibody was used to clearly identify these large mononuclear cells and to determine if different numbers of macrophages that persisted within or were recruited to the Peyer's patches of mice infected with various invasion mutants could be detected. Duplicate sections were incubated with both antibodies to determine if there was a correlation between the presence of macrophages and the location of pathology. The antibody used for this study was a general anti-mouse macrophage antibody capable of recognizing the entire population of mouse macrophages. Since ileal Peyer's patch macrophages are few in number (44), background staining was not problematic (data not shown).

Peyer's patches from both 8081v- and *ail* mutant-infected mice exhibited positive staining for the anti-invasin antibody (Fig. 4B and data not shown). The staining pattern was most pronounced in necrotic and surrounding areas and dispersed

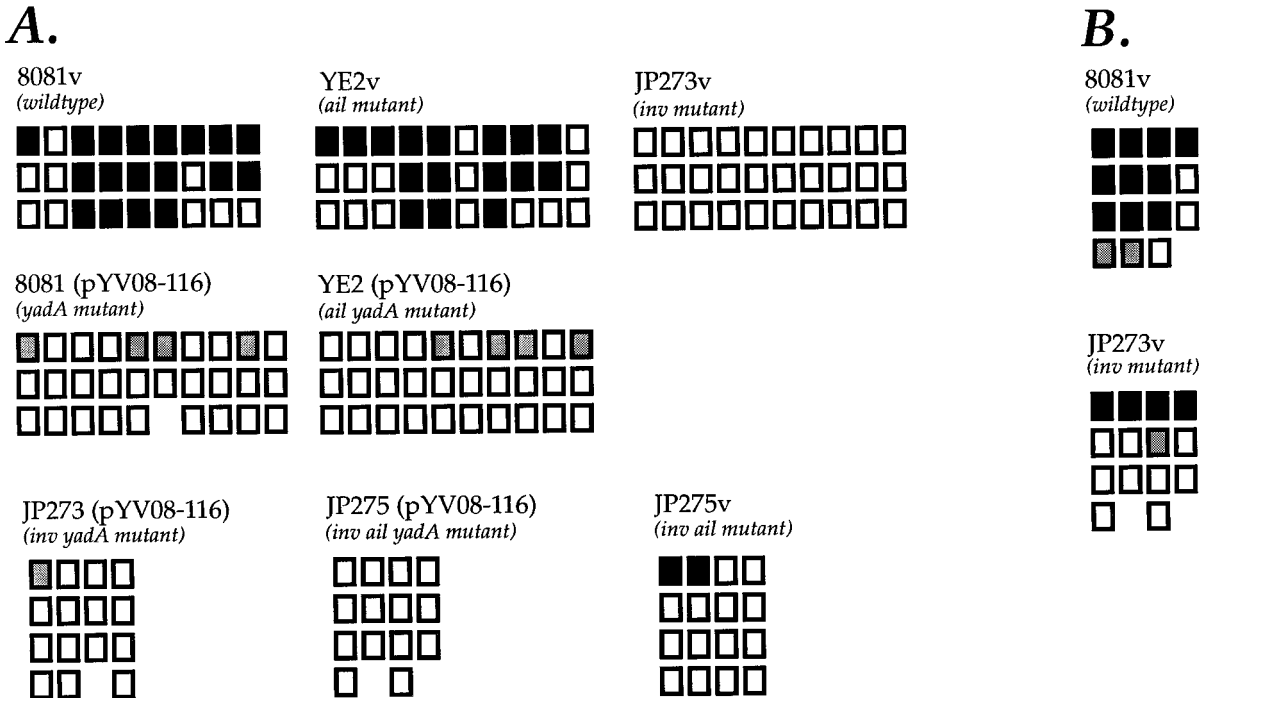

FIG. 3. Pathology exhibited by murine Peyer's patches infected with various *Y. enterocolitica* mutants. BALB/c mice were infected i.g. with approximately 5×10^8 bacteria, and the Peyer's patches were harvested at 42 (A) and 72 (B) h postinfection. Tissues were stained with hematoxylin and eosin as described in Materials and Methods. Tissues were scored individually in a blind study for the presence of inflammation and necrosis (solid boxes) and inflammation only (shaded boxes) as indicated. Vertical columns indicate the numbers of Peyer's patches isolated from individual mice, and horizontal rows indicate Peyer's patches isolated from different mice.

out from these foci, confirming bacterial presence centrally in the microabscesses. This result was consistent with the Gram and Steiner-Steiner staining patterns (data not shown). Single large mononuclear cells adjacent to these sites also showed dense cytoplasmic staining, suggestive of an intracellular location for bacteria more peripheral to necrotic foci. These same type of cells also stained positively with the anti-mouse macrophage antibody (Fig. 4C and data not shown). These results suggest that *Y. enterocolitica* organisms were ingested by macrophages; however, bacterial viability could not be determined. Intense staining of the anti-mouse macrophage antibody occurred only in follicles containing inflammation and necrosis; follicles free of inflammation and necrosis within a given sample remained unstained. Thus, mouse macrophages appeared to persist near or be recruited specifically to sites where bacteria were found within Peyer's patches.

The *yadA*- and *ail yadA*-infected Peyer's patches both stained positively for the anti-invasin antibody, even though no necrotic foci were observed in these samples. The staining pattern was not as centralized in these samples as those in the wild type- or *ail* mutant-infected tissues, in agreement with the inflammatory changes seen with the hematoxylin-eosin samples. The anti-mouse macrophage antibody positively stained regions of samples from tissues infected with the *yadA* and *ail yadA* mutants (Fig. 4D and E), though the staining was diminished compared with that of the previous two samples of tissues infected with either the wild-type strain or the *ail* mutant. Upon arrival at the Peyer's patches, circulating macrophages enter the periphery of the follicles, after which they proceed to the inner parenchyma region. Both of these samples showed peripheral macrophage staining, possibly indicating that macrophage recruitment is at an earlier stage, has been delayed, or has not persisted. Comparison of these two mutants revealed that the *yadA* mutant had a more advanced inflammatory process and displayed larger inflammatory centers and a more focused macrophage recruitment or persistence than the *ail yadA* mutant, which showed more peripheral macrophage staining.

Peyer's patches from mice infected with the *inv* mutant were negative for staining with both antibodies at 42 h postinfection, indicating that a detectable number of bacteria was not found in these samples at this time point, in agreement with the lack of inflammation observed (Fig. 4F and data not shown). However, the anti-invasin antibody did detect the presence of bacteria in Peyer's patches at 72 h postinfection (Fig. 4G). The *inv* mutant was more diffusely spread throughout the Peyer's patches compared with the wild type, which had a more focused pathology (Fig. 4H). This observation shows that *inv* mutants are eventually able to penetrate the Peyer's patches in detectable numbers, which is supported by earlier observations (63) and the histology discussed above.

DISCUSSION

After being ingested, *Y. enterocolitica* rapidly reaches the Peyer's patches of the terminal ileum. Studies of tissue from infected animals suggest that the bacteria accomplish this by passing through the cells of the intestinal epithelium (27, 29). Although this sequence of events is well documented, the molecular mechanisms by which *Y. enterocolitica* progresses through each step are just beginning to be understood. *Y. enterocolitica* produces several adhesins that could promote attachment to the ileal wall, but it requires invasin to efficiently penetrate the intestinal barrier to reach the lamina propria (63). The present study was initiated to determine whether or not other known *Y. enterocolitica* invasion factors (Ail and

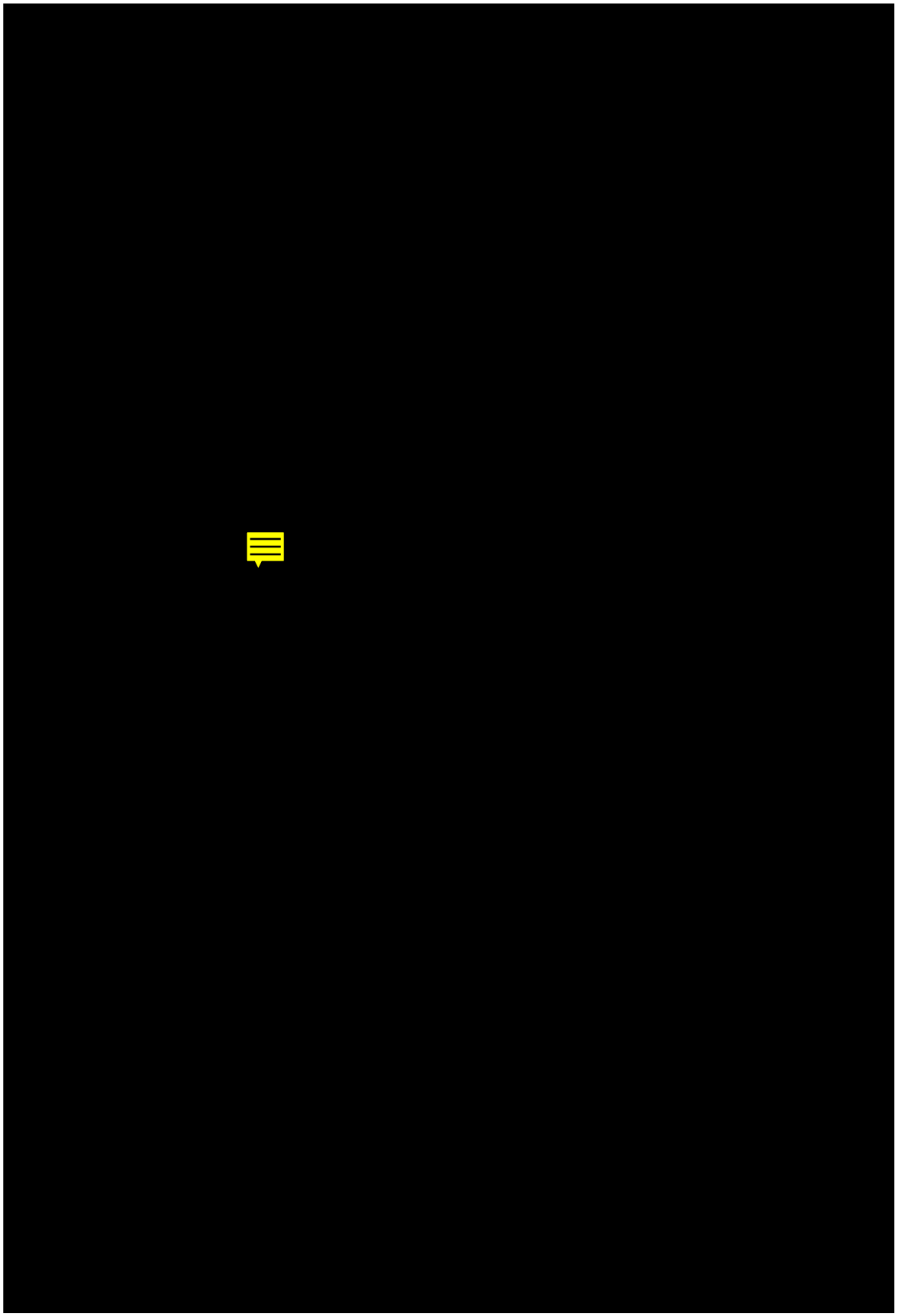

FIG. 4. Immunohistochemical staining of murine Peyer's patches infected with *Y. enterocolitica* mutants. BALB/c mice were infected with various *Y. enterocolitica* invasion or survival mutants as indicated. Mice were infected i.g. with approximately 5×10^8 bacteria, and the Peyer's patches were harvested at 42 and 72 h postinfection. Tissues were incubated with an anti-invasin polyclonal antibody or an anti-mouse macrophage polyclonal antibody as described in Materials and Methods. A sample from a mock-infected mouse is shown (A). The Peyer's patches of this mouse were harvested at the same time as the 42-h wild-type sample. (A) Negative control (mock-infected mouse) with anti-invasin antibody. Magnification, ×5.9. (B) Wild type at 42 h postinfection with anti-invasin antibody. Magnification, ca. ×2.4. (C) Wild type at 42 h postinfection with anti-mouse macrophage antibody. Note the confluent band of macrophage staining around the inflammatory focus seen in panel B. The anti-mouse macrophage antibody shows characteristic peripheral staining of the surface of individual macrophages. Magnification, ca. ×2.4. (D) *yadA* mutant at 42 h postinfection with anti-mouse macrophage antibody. The arrowhead shows deeper parenchymal staining compared with that with the *ail yadA* mutant (see panel E). Magnification, ×5.9. (E) *ail yadA* mutant at 42 h postinfection with anti-mouse macrophage antibody. The arrowhead shows peripheral staining of macrophages. Magnification, ×5.9. (F) *inv* mutant at 42 h postinfection with anti-invasin antibody. Magnification, ×5.9. (G) *inv* mutant at 72 h postinfection with anti-invasin antibody. Magnification, ca. ×2.4. (H) Wild type at 72 h postinfection with anti-invasin antibody. Magnification, ca. ×2.4.

YadA) could promote uptake of the bacteria in the absence of invasin and to characterize the pathology observed in mice infected with various mutants.

Effect of mutations in *inv*, *ail*, and *yadA* on early events and virulence. As was previously demonstrated for *Y. pseudotuberculosis* (12, 89), we found that YadA provides *Y. enterocolitica* with the potential to be internalized by tissue culture cells. However, this invasion pathway was only detectable in an *inv* mutant background and only compensated for ~2% of the invasin defect. YadA is maximally expressed at 37°C (41), but it promoted uptake of *Y. enterocolitica* grown at 23°C. This may have been due to low-level expression of YadA in *Y. enterocolitica* cultures grown at 23°C (76), and *yadA* may have been induced during the 37°C incubation of the tissue culture invasion assay. This low-level invasion is even more difficult to assess with *Y. enterocolitica* grown at 37°C and harboring pYV, because this plasmid produced cytotoxic factors at 37°C (65). In fact, uptake of the *inv* mutant grown at 37°C was abolished when this strain carried pYV (data not shown).

In vivo, *Y. enterocolitica inv* mutants showed delayed colonization of Peyer's patches in some mice which could have been mediated by Ail and/or YadA. *Y. enterocolitica ail*, *yadA*, and *ail yadA* mutants penetrated the mouse intestinal mucosa as well as the wild type early after infection, but within 2 days the *yadA* mutants were beginning to be cleared and the *ail yadA* double mutant was essentially eliminated. The *yadA* mutants are avirulent, probably because they were unable to survive the host defenses. YadA has been shown to be antiphagocytic (32, 69), and it has been suggested that it is important for delivery of cytotoxic factors YopE and YopH to target cells (12, 71). Thus, the *yadA* mutant may not be able to resist phagocytosis and killing by the neutrophils and macrophages that are found at the sites of infection. It is worthy to note that Ail and YadA are both capable of protecting *Y. enterocolitica* from the bactericidal effects of normal human serum (9, 13, 46, 59, 64); however, it is unlikely this is a factor in this animal model, since mouse serum is not bactericidal for *Y. enterocolitica* (30, 85).

We were able to complement the persistence defect of the *yadA* mutant by providing *yadA* in *trans*, but we were unable to complement the *yadA ail* mutant by examining an *ail* merodiploid. The failure of *ail* to complement this strain suggests that the chromosomal region containing the rearrangement upstream of the *ail* mutation (85) contributes to bacterial persistence in the Peyer's patches. This rearrangement could disrupt a previously unidentified virulence gene, prevent expression of proteins that interact with Ail, or disrupt regulatory factors necessary for proper Ail expression. The last possibility is unlikely, since Western blot analysis shows that the amount of Ail produced by the wild type is similar to that produced by the *ail* merodiploid *yadA* mutant, YE3(pYV08-116).

The role of *inv*, *ail*, and *yadA* during a *Y. pseudotuberculosis* infection has not been studied in as much detail as that in *Y. enterocolitica*; however, the virulence attributes of these two species are manifestly different. *Y. pseudotuberculosis* appears to have an *ail* homolog, but preliminary evidence indicates that it does not function to promote invasion (89). Kapperud et al. (37) found that a *Y. enterocolitica yadA* mutant was significantly reduced in its capacity to colonize the ileum of orogastrically infected mice, and here we show that *yadA* is an essential virulence determinant of *Y. enterocolitica* by either the oral or i.p. route of infection. In contrast, a *yadA* mutation has no effect on the virulence of *Y. pseudotuberculosis* (15, 72) or on its ability to colonize the mouse intestine (37). Interestingly, a *Y. pseudotuberculosis inv yadA* double mutant was more virulent than the parent strain (72), but we found the *Y. enterocolitica inv yadA* double mutant to be avirulent. Although *Y. enterocolitica* and *Y. pseudotuberculosis* cause similar disease syndromes and clearly share many of the same virulence factors, one must be careful about extrapolating results for *Yersinia* species.

With these and other results taken into account, a working model for intestinal invasion by *Y. enterocolitica* can be viewed as follows. Upon arrival at the terminal ileum, invasin is the primary factor necessary for initial penetration. However, other factors are able to substitute in the absence of invasin, since *inv* mutants penetrate at a lower efficiency. Delayed entry of an *inv* mutant may be due to nonspecific host factors, such as sampling of the luminal contents by M cells, and bacterial factors may include an as yet unidentified invasion factor that is only expressed in vivo. Alternatively, entry may occur after bacterial replication causes damage to epithelial cells; thus, *Y. enterocolitica* can eventually reach the lymphatic system or bloodstream directly. However, histological examination of tissue samples showed no evidence of significant damage to the epithelium. The absence of delayed colonization by the *inv yadA* double mutant indicates that YadA may play an accessory role in the initiation of uptake. However, the persistence defect associated with the *yadA* mutation makes it difficult to definitively make this conclusion. Both YadA and a region near *ail* appear to promote persistence of the bacteria after initial colonization of the Peyer's patches. YadA may protect *Y. enterocolitica* from killing by PMNs (19) as well as promote cellular attachment for the delivery of the YopE and YopH cytotoxins (12, 71).

Histopathological analysis of infections caused by *Y. enterocolitica* invasion mutants. A second aim of this study was to examine the histopathology of murine Peyer's patches after infection with various *Y. enterocolitica* mutants so that the bacteria could be localized within the Peyer's patches and the primary management of *Y. enterocolitica* infection in the Peyer's patches by unstimulated circulating macrophages could be characterized. The bowel epithelium of all samples displayed normal architecture, suggesting that the invasion process of *Y. enterocolitica* does not involve the alteration of the intestinal epithelium. In general, the severity of inflammation caused by the *Y. enterocolitica* mutants was consistent with the number of bacteria colonizing the Peyer's patches. Immunohistochemical staining of our *Y. enterocolitica*-infected samples with the anti-invasin antibody, a general marker for *Y. enterocolitica*, corre-

sponded with the pathology observed by hematoxylin-eosin staining. Staining with anti-mouse macrophage antibody confirmed macrophage recruitment or retention specifically to severely infected areas within the Peyer's patches.

The *ail* mutant (which has a phenotype similar to that of the wild type in murine oral LD_{50} experiments, kinetics experiments, and invasion of Peyer's patches [85]) displayed a pathology similar to that of the wild-type strain. This mutant also displayed staining patterns with the anti-invasin antibody similar to those in wild type-infected tissues. At 42 h postinfection, the *ail yadA* mutant caused a pathology similar to that caused by the *yadA* mutant, which persists better within the Peyer's patches. The number of bacteria present may have been below a threshold needed to produce both inflammation and necrosis. If so, this threshold may lie between 2.8×10^5 (*yadA*) and 3.4×10^7 (*ail*) CFU. Alternatively, the bacteria may need to persist in the Peyer's patches longer than the *yadA* mutant, which begins to die between 18 and 42 h postinfection. Incubation of *yadA*-infected tissues with the anti-mouse macrophage antibody showed a more progressed inflammatory response at 42 h postinfection compared with that of the *ail yadA* mutant. This result shows that the *yadA* mutant is capable of provoking a more extensive immune response than the *ail yadA* mutant at this time point and is consistent with the numbers of bacteria observed in the Peyer's patches.

Our results confirmed those of Pepe and Miller (63) regarding the necessity of invasin for penetration of the intestinal epithelium. Though the Peyer's patches of some mice infected with the *inv* mutant show high bacterial numbers at 42 h postinfection (63), our experiments showed that a population of 10 mice infected with this mutant had no detectable inflammation at this time point. Previous studies showed that approximately half the mice infected with the *inv* mutant had nearly wild-type levels of bacteria in their Peyer's patches. This number exceeds the hypothetical threshold suggested by experiments with the *yadA* and *ail yadA* mutants; however, it may be that this threshold must be maintained for a period of time for pathology to be visualized by our methods. At 72 h postinfection, one-third of the Peyer's patches examined showed pathological changes. Therefore, it appears that pathology does progress in the Peyer's patches of mice infected with the *inv* mutant, albeit with delayed kinetics. The *inv* mutant was not detectable by immunohistochemistry in the Peyer's patches until 72 h postinfection and was more diffusely spread within the follicles than the wild-type strain.

To date, there are few data in the literature dealing with the cellular immune response mounted against *Y. enterocolitica*. Lymphoid cells transferred from the Peyer's patches of a *Y. enterocolitica* (serotype 03)-infected mouse were protective against *Yersinia pestis* (1). T cells have been implicated in combating infection with *Yersinia* spp., since thymus-bearing $(C57BL/6 \times BALB/c)F_1$ mice were more resistant to infection with *Yersinia* spp. than their athymic (nude) littermates (28). Other studies have strongly suggested a role for T cells and mediators gamma interferon and tumor necrosis factor alpha (2–7, 14).

Immunohistological studies of *Yersinia*-infected mice confirmed the presence of T cells in liver granuloma-like lesions of intravenously infected mice after 3 days of infection; T cells increased in number until 5 days postinfection (4). Granulomatous lesions are thought to be caused by facultative intracellular pathogens such as *Listeria monocytogenes*, which is eliminated by macrophages previously activated by specific T lymphocytes (47, 48, 54, 55). In contrast to granulomas, pyogenic lesions are thought to result from infection with extracellular pathogens that are eliminated primarily by nonspecific cellular mechanisms, such as PMNs and unstimulated circulating macrophages. Although *Y. enterocolitica* has been found extracellularly in vivo (27, 29, 30, 43, 84), it may not be exclusively extracellular, since *Y. enterocolitica* also has been observed multiplying within mononuclear cells of infected rabbits (80, 82). *Y. enterocolitica* has many defenses against phagocytic cells, and it may be that only activated macrophages are able to contain and eliminate these bacteria, thus explaining the necessity for an active T-cell response.

In contrast to the studies by Autenrieth et al. (4), who saw granuloma-like lesions in the livers of intravenously infected C57BL/6 mice 5 days postinfection, previous studies of orally infected CD-1 mice found no evidence of granulomas but instead reported necrotic lesions containing large numbers of PMNs and some monocytes (18). This type of pathology closely resembles what has been observed in infections of humans (17, 83). The results of our examination of orally infected BALB/c mice more closely resemble those of Carter (18). However, our examination was done only at early time points, and this could explain why our results differ from those of Autenrieth et al. (4). These differences could also be explained by the difference in the route of inoculation and the difference in the strains of mice used, since C57BL/6 mice are relatively resistant to infection with *Y. enterocolitica* (28).

We did observe bacterial staining in the cytoplasm of large mononuclear cells in Peyer's patches. These same tissue samples stained positively with the anti-mouse macrophage antibody in these locations, suggesting that bacteria were located within macrophages. Interestingly, potentially intracellular bacteria were observed in the Peyer's patches of wild type- or *ail* mutant-infected mice, the strains that cause the most pathology in mice. It is possible that the other mutants were intracellular as well but that our assay conditions were not sensitive enough to detect them. As stated above, the intracellular location of *Y. enterocolitica* in vivo is controversial (27, 29, 30, 43, 80, 82, 84). It was not clear if the bacteria that appeared to be within macrophages were in the process of being degraded or if they were surviving, in which case they may play an important role during the infection. Resolution of this point awaits further investigation.

ACKNOWLEDGMENTS

The first two authors contributed equally to this study.

We thank Mikael Skurnik for plasmids pYV08-116 and pYV085, M. John Pickett for the use of his laboratory space, and S. Kinder for critical reading of the manuscript. We thank Ana Maria Zaragoza, Saeedeh Shapourifar-Tehrani, and Isabelle Rose (Autopsy-Histology Laboratory, Department of Pathology and Laboratory Medicine, UCLA) for assistance in the histological preparation of specimens and Lorna Cheng (Immunohistochemistry Laboratory, Department of Pathology and Laboratory Medicine, UCLA) for assistance in the preparation of immunohistochemical preparations.

This work was supported by Public Health Service National Research Award GM07104 to J.C.P. and by National Institutes of Health grants AI27342 and AI01230 to V.L.M., who is a Pew Scholar in the Biomedical Sciences.

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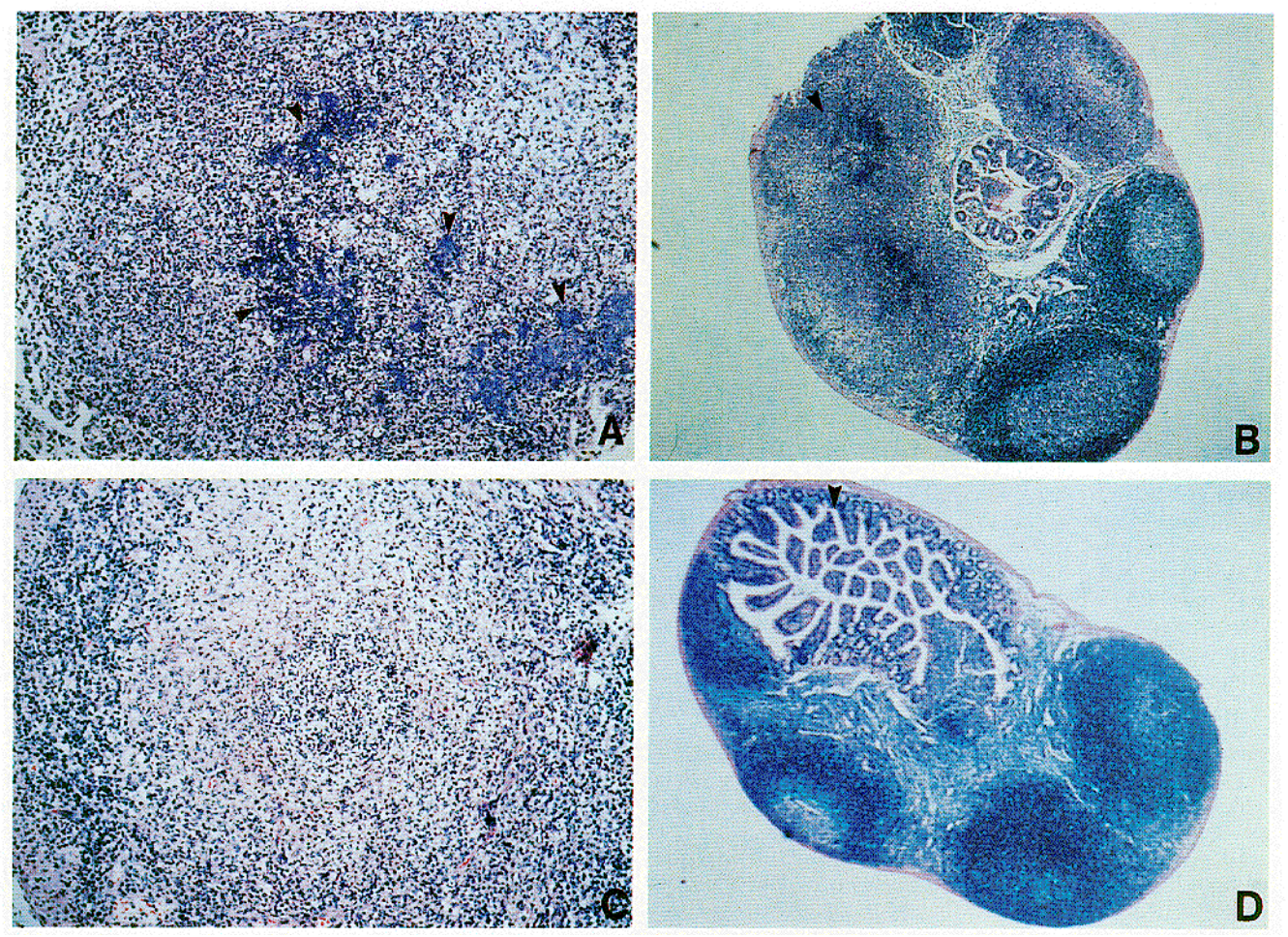
Editor: B. I. Eisenstein.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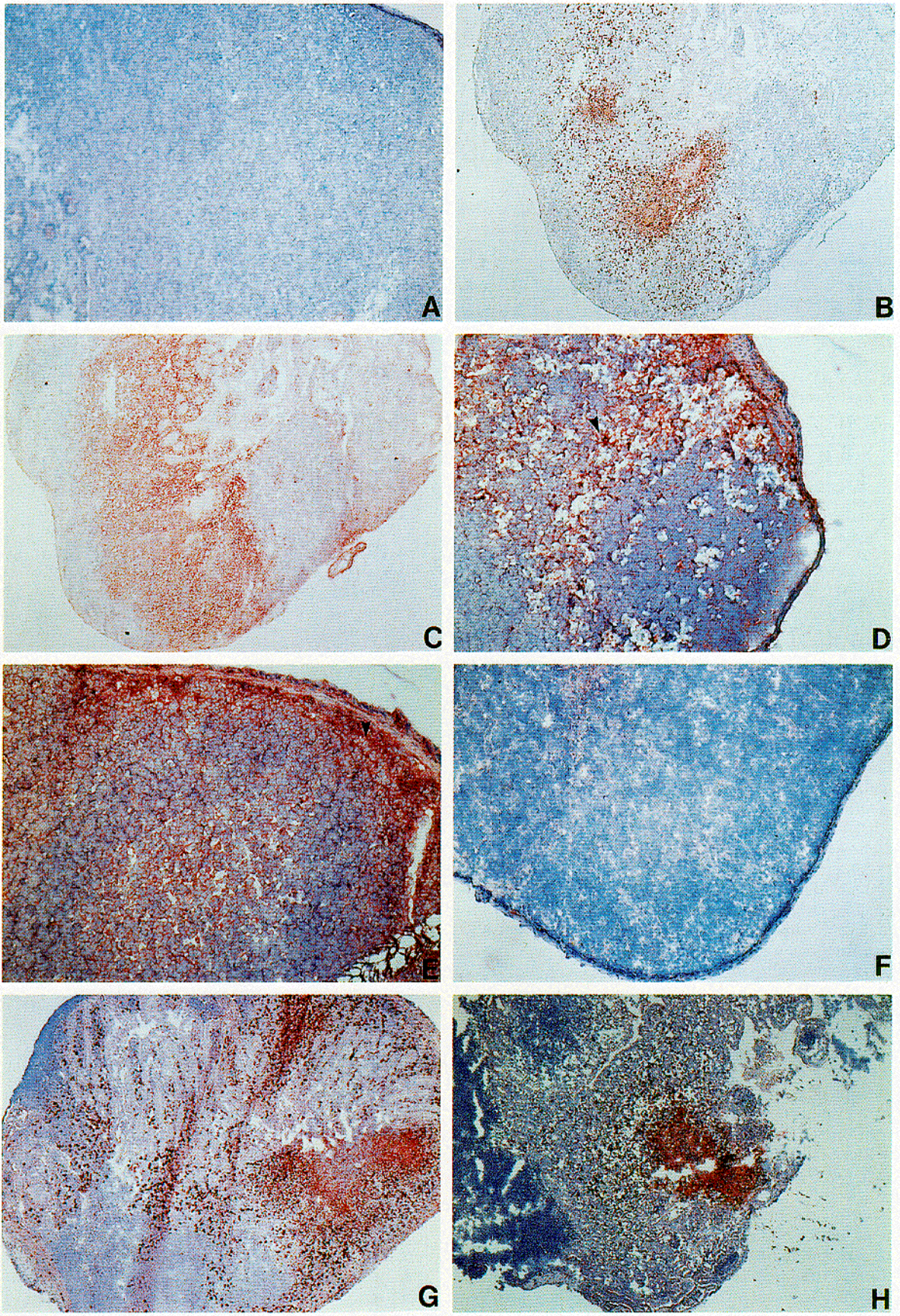
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