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VIRULENCE MECHANISMS OF BACTERIAL PATHOGENS
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*Chapter 4*

# Virulence Mechanisms of Enteroinvasive Pathogens

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## INTRODUCTION

Enteroinvasive bacterial pathogens which cause disease in animals include members of the genera *Salmonella*, *Yersinia*, and *Shigella*. *Salmonella* infections in cattle, sheep, swine, and horses are usually manifested as acute or chronic enteritis, but acute bacteremia accompanied by abortion and death can also result from ingestion of these organisms. *Yersinia pseudotuberculosis* is a common cause of epizootic disease of birds and rodents, and this organism can also cause enteritis and septic abortions in domestic animals. Humans are the natural reservoirs of *Shigella* species, but *Shigella* infections often occur in captive monkey populations.

## LABORATORY MODELS

Several laboratory models have been useful in the study of enteroinvasive enteric diseases, and a brief description of these will provide the background for a discussion of the pathogenesis of the infections. The least stringent model is an in vitro system which uses cultured mammalian cells as surrogates of intestinal epithelial cells. Since virulent *Shigella*, *Salmonella*, and *Yersinia* strains invade these cells by a process which appears to be analogous to the invasion of enterocytes in vivo, this model has been useful in evaluating one aspect of the virulence of individual bacterial strains and in studying the mechanism of bacterial invasion. Although the tissue culture model can assess the ability of an enteroinvasive strain to initiate infection, it does not address other determinants of virulence. For example, the organisms must overcome host defense mechanisms in the intact intestine which are not present in vitro. Therefore, various animal models have been used in the study of enteroinvasive pathogens. The ability of shigellae to invade corneal epithelial cells and elicit keratoconjunctivitis in the eyes of rabbits, guinea pigs, or mice (Sereny test) assesses both invasiveness and resistance to mucosal defense mechanisms. The ligated rabbit ileal loop model has been used to assess the ability of shigellae or salmonellae to invade the intestinal mucosa and elicit inflammation and fluid secretion. Oral challenge of starved and opiated guinea pigs measures the lethality of these organisms in a compromised host, whereas oral challenge of monkeys can reproduce the enteritis of *Salmonella* infections or the dysentery commonly seen with shigellosis.

## OVERVIEW OF PATHOGENIC MECHANISMS OF *SHIGELLA* SPP.

### Ingestion

In humans, as few as 10 shigellae can cause dysentery, whereas the 50% infective dose in monkeys is approximately $10^{10}$. The basis of the relative resistance of monkeys to *Shigella* infection is unknown. Shigellae do not colonize or invade the small intestine unless peristaltic motion is inhibited by opiates or ligation. The lack of chemotactic motility may be one mitigating factor which discourages colonization of the small intestine by shigellae, but recent data indicate that pancreatic enzymes may also protect the mucosa from invasion by shigellae. Pretreatment of a virulent culture of *Shigella flexneri* 5 with trypsin or chymotrypsin causes the loss of invasive potential as measured by the ability of these organisms to invade HeLa cell monolayers in vitro. Suspension of the organisms in fresh culture medium allows the complete regeneration of the invasive phenotype after two rounds of cell division (T. L. Hale, manuscript in preparation). These data suggest that outer membrane proteins which are necessary for adherence and invasion are cleaved as shigellae pass through the duodenum and jejunum. Presumably these outer membrane proteins are regenerated in time to facilitate invasion of the colonic epithelium, which is the target tissue of shigellae.

### Diarrheal Stage

Although they do not actually invade the mucosa, shigellae often elicit abnormal fluid secretion when traversing the small intestine. For example, there is net fluid secretion in the jejunum when rhesus monkeys ingest virulent *S. flexneri*. In combination with inhibited colonic absorption, this abnormal fluid flux is manifested as diarrhea. If the shigellae are injected directly into the cecum, however, the only transport abnormality is the inhibition of fluid absorption, and the clinical manifestation is dysentery (36). These data suggest that an enterotoxin is elaborated by shigellae during transit through the ileum, and the obvious candidates for such a toxin are the Shiga toxin produced by *Shigella dysenteriae* 1 or the Shiga-like toxin produced by both *S. dysenteriae* 1 and the other *Shigella* spp. (32). The latter toxin is neutralizable by antibody raised against the classical Shiga toxin, but the Shiga-like toxin genes share only about 50% homology with the Shiga toxin genes. Arguing against a role for these toxins in the diarrheal stage of disease is the experimental finding that ingestion of a noninvasive strain of *S. dysenteriae* 1 does not cause diarrhea in monkeys, even though this strain is highly toxigenic (9). In the final analysis, invasion of the intestinal mucosa seems to be the essential step in the pathogenesis of shigellosis, and the additional role of *Shigella* toxins remains unclear.

Once shigellae have traversed the upper digestive tract, they encounter fatty acids and reducing conditions in the colonic lumen. These by-products of the metabolic activity of fusiform anaerobes are toxic for shigellae. In addition, chemostat experiments designed to simulate the luminal environment in vitro indicate that the most serious obstacle facing members of the family *Enterobacteriaceae* attempting to colonize the bowel is the competition of the resident flora for carbon sources utilizable under low-pH and reducing conditions (19). These environmental pressures have apparently selected and maintained the invasive phenotype in the genus *Shigella*. The evolution of this phenotype has allowed shigellae to escape from the fully occupied niche of the lumen and to occupy the extreme environment represented by the cytosol of the colonic epithelial cells (28). By occupying this unique environmental niche, shigellae also avail themselves of an inexhaustible carbon source—glucose from the host blood stream.

### Colonic Invasion and Dysentery

The intestinal mucus layer is the first barrier encountered by shigellae which are invading the colonic mucosa. The effectiveness of mucus as a barrier can be readily demonstrated

in vitro by using cultured explants of monkey colon. These explants, which are characteristically blanketed by a layer of mucus, cannot be infected by a pure culture of *S. flexneri* (G. Dinari and T. L. Hale, unpublished observations). Since solubilized monkey mucus does not inhibit the invasion of epithelial cells by shigellae (5), the mucus on the explants probably acts as a mechanical barrier. Other investigators have found that *S. flexneri* will not attach to intestinal slices obtained from gnotobiotic mice; however, these organisms do attach to slices which have been treated with extracts of normal mouse feces (34). Thus, it appears that glycosidases produced by the normal intestinal flora may indirectly facilitate *Shigella* invasion by digesting colonic mucus.

Once they have breached the mucus layer, shigellae apparently induce endocytic activity in the normally nonphagocytic enterocytes. The tissue culture model involving the use of HeLa cells has been used to characterize this phenomenon. The first step of this process involves adherence of shigellae to the HeLa cell membrane. This adherence is mediated by components which can be cleaved by trypsin treatment of intact bacteria, so that it is apparently different from the lipopolysaccharide-mediated adherence to guinea pig enterocytes which has previously been reported (21).

Shigellae invade HeLa cells by an endocytic process which does not rupture the plasma membrane and which requires both host cell energy production and microfilament function (13). Ultrastructurally, the invasion process is characterized by the formation of areas of close apposition between the surface of the bacterium and the surface of the host cell (13). These areas of close apposition may represent localized binding of bacterial surface ligands to receptors on the host cell membrane. Occasionally a thin section reveals a number of areas of close apposition around the circumference of an attached organism (13, 17), and this morphology gives the impression that the shigellae are "zippered up" into an endocytic vacuole by sequential receptor-ligand binding.

When shigellae are internalized by HeLa cells, they are initially contained within endocytic vacuoles. Within 30 min, however, these vacuoles disappear (13, 41). A similar dissolution of endocytic vacuoles apparently occurs within infected colonic epithelial cells, because membrane-bound shigellae are rarely observed in vivo (44). Organisms which are unable to lyse these endocytic vacuoles are less virulent because they are less able to spread to contiguous epithelial cells (41). Another intracellular event which accompanies the endocytosis of virulent shigellae is the inhibition of host cell protein synthesis (41). The cytotoxic manifestation of Shiga-like toxins probably inhibits macromolecular synthesis (14), but the lysis of microsomal membranes may also be involved (41). Glucose and amino acid transport continue in infected HeLa cells, but these nutrients support bacterial multiplication rather than host cell functions (14, 31). Presumably a similar parasitic relationship allows shigellae to survive and propagate within the colonic epithelium, and, as mentioned above, this is probably the powerful selective advantage which has allowed the evolution and conservation of the invasive phenotype.

A secondary result of this parasitism of the colonic mucosa is the tissue destruction which is the underlying cause of the symptoms of bacillary dysentery. The inflammation elicited by mucosal destruction is accompanied by increased peristaltic motion and goblet cell evacuation. These abnormal bowel functions have the effect of sweeping shigellae that have been released from degenerating enterocytes out of the intestinal tract, and this discourages the establishment of new foci of infection.

## GENETICS OF VIRULENCE IN SHIGELLAE

### Chromosomal Virulence Genes

The virulence-associated regions of the chromosome in *S. flexneri* 2a have been extensively studied. These studies, which involve the conjugal transfer of chromosomal material from *Escherichia coli* K-12 to *S. flexneri* by uninter-

rupted matings, show that replacement of three *Shigella* chromosomal regions with homologous regions from *E. coli* has an effect upon the virulence of the *Shigella* recipient in animal models. For example, incorporation of a large chromosomal region including the *xyl* (79 min) and *rha* (88 min) genes is associated with loss of ability to cause a fatal infection in orally challenged guinea pigs (7), and replacement of the *his* locus (45 min) is associated with loss of the ability to cause a positive Sereny test (8). Transduction of the *purE* locus (12 min) from *E. coli* to *S. flexneri* also results in both a Sereny-negative phenotype and a decrease in virulence in the guinea pig model (6). Since none of these avirulent *E. coli-S. flexneri* hybrids lost the ability to invade mammalian cells in vitro, it appears that the chromosomal regions replaced by *E. coli* genes are necessary for the survival of shigellae in the lumen of the bowel or in the tissues.

Potential virulence determinants have been identified in two of these *Shigella* chromosomal regions. The *xyl-rha* region encodes an aerobactin iron-binding system in *S. flexneri* (11); however, recent analysis of aerobactin-negative *Shigella* mutants indicates that this phenotype is not necessary for virulence (26). The ability to elicit fluid accumulation in the ligated rabbit ileal loop model is associated with the chromosomal region including *mtl* (80 min) and *arg* (90 min) (39). The genes encoding a Shiga-like toxin have been localized in this chromosomal region in *S. dysenteriae* 1 (46), and the virulence determinant encoded by this region in *S. flexneri* may be a toxin (32). The *his* genes are linked to the *rfb* gene cluster in *E. coli*, and this chromosomal region is necessary for synthesis of the complete somatic antigen in either *E. coli* or *S. flexneri*. Although the somatic antigen is probably the key virulence determinant encoded by the *his* region in *Shigella* species, an additional determinant(s) may also be expressed (8). In contrast to the *xyl-rha* and the *his* regions, a virulence determinant encoded by the *purE* region has not been identified. Nonetheless, this region has been given a genetic epitaph based on the Sereny test phenotype, i.e., *kcp*, denoting keratoconjuctivitis provocation (6).

## Plasmid Virulence Genes

Virtually all selectable chromosomal markers can be conjugally transferred from *S. flexneri* to *E. coli* K-12 without altering the avirulent phenotype of the recipient. This observation suggests that extrachromosomal elements are also necessary for expression of virulence. About 5 years ago it was shown that a 120-megadalton (MDa) plasmid was indeed necessary for expression of both the group D somatic antigen and the invasive phenotype in *Shigella sonnei* (40). Later it was found that expression of the latter phenotype was associated with a 140-MDa plasmid in other *Shigella* species and in enteroinvasive strains of *E. coli* (38). These plasmids are at least 80% homologous and are functionally interchangeable in their ability to confer the invasive phenotype (38). A 6-MDa plasmid, which is unrelated to the invasive plasmids, is necessary for synthesis of the complete somatic antigen in *S. dysenteriae* 1 (49).

Genetic analysis of the large plasmids of *S. flexneri* and *S. sonnei* has revealed at least four loci which influence various aspects of virulence. For example, a 22-MDa fragment of the 140-MDa plasmid of *S. flexneri* 5 can confer the invasive phenotype upon a *Shigella* strain which has lost the 140-MDa plasmid (27). Transposon mutagenesis indicates that this region of *Shigella* invasion plasmids carries at least two invasion loci, designated *ipa* (invasion plasmid antigen) (3) and *invA* (48). Two other loci, which are apparently located outside the 22-MDa invasion region, may also be necessary for expression of the virulent phenotype. These include the *virF* genes, which are necessary for the binding of Congo red (37), and the *virG* locus, which is necessary for expression of a positive Sereny phenotype (42).

Three *ipa* gene protein products have been designated *b* (57 kDa), *c* (43 kDa), and *d* (37 kDa) (3, 15). Additional products which are also encoded by *ipa*-linked genes include polypeptides designated *a* (78 kDa), *f* (25 kDa), and *g* (20 kDa) (15). *ipa* gene products *a* through *d* are immunodominant protein antigens which elicit serum and mucosal antibody during *Shi-*

*gella* infections in monkeys (4, 30) and humans (16). Four *inv* gene products, of 81, 47, 41, and 38 kDa, have been identified in minicells (48). These proteins, which are immunologically distinct from the *ipa* gene products, do not induce antibody in convalescent-monkey antisera (T. L. Hale and H. Watanabe, unpublished data). The *virF* region expresses three polypeptides, of 21, 27, and 30 kDa (42). The products of *virG* have not yet been identified.

### Possible Roles of Plasmid Gene Products in Virulence

Anucleate minicells isolated from *S. flexneri* can invade HeLa cells (15). Since minicells contain no chromosomal DNA, the 140-MDa plasmid apparently carries all the genetic information necessary for expression of the invasive phenotype. As discussed above, a 22-MDa fragment of this plasmid has now been shown to carry the genes necessary for the invasion step, and some of the protein products of these genes have been identified. The functions of these proteins have not been rigorously defined, but preliminary data suggest that the *ipa* gene polypeptides designated *b* and *c* are probably the bacterial proteins which actually trigger the uptake of shigellae. When extracted under nondenaturing conditions, these polypeptides have a proclivity for the surface of HeLa cells. It is our current hypothesis that this interaction triggers endocytic activity, which results in the ingestion of the attached bacterium. The *invA* locus seems to function as a transport system, allowing insertion of *ipa* polypeptides into the outer membrane in a functional orientation (T. Pal and T. L. Hale, unpublished data).

## OVERVIEW OF PATHOGENIC MECHANISMS OF *SALMONELLA* AND *YERSINIA* SPP.

### Ingestion

In humans the infective dose of *Salmonella typhimurium* is approximately $10^5$, while ingestion of $10^{10}$ organisms can cause gastroenteritis in monkeys. Rough *Salmonella* strains are avirulent, and the underlying cause of avirulence probably involves the loss of chemotactic motility as well as diminished resistance to gastric acidity (29). Smooth strains, which are more actively motile than rough strains, are attracted to damaged HeLa cells in vitro by a gradient of the amino acid glycine (47). It has been proposed that *S. typhimurium* organisms are attracted to the dying cells on the villus tips of the ileal epithelium and that this chemotactic response facilitates the establishment of a *Salmonella* infection (47). The action of pancreatic enzymes may also enhance the invasiveness of the salmonellae. Pretreatment of *S. typhimurium* with trypsin causes these organisms to invade HeLa cells much more avidly than do untreated control cultures (Hale, unpublished data). Thus, the enzymatic environment of the small intestine may activate the invasive phenotype by modifying protein components of the outer membrane. The possible roles of mannose-resistant hemagglutinins and mannose-sensitive hemagglutinins (type 1 fimbriae) in mediating the adherence of *S. typhimurium* to cultured epithelial cells in vitro has been documented, but there is no clear evidence that these adhesins play a role in attachment to the intestinal epithelium (45).

Once the salmonellae have penetrated the mucus layer of the small intestine, the microvilli on epithelial cells in the immediate vicinity degenerate. Invaginations then form in the apical cytoplasm of enterocytes, and the organisms are taken up within endocytic vacuoles (43). The morphological and biochemical aspects of this invasion process have been studied in the HeLa cell model, and the general pattern is similar to that observed with shigellae. The organisms adhere to the plasma membrane and develop areas of close apposition which may represent sequential receptor-ligand binding (23). Ingestion of attached organisms requires host cell energy production and microfilament contraction (24). Unlike shigellae, however, the ingested salmonellae remain enveloped within endocytic vacuoles in the cytoplasm of HeLa cells (23) or enterocytes (43). Indeed, many

*Salmonella* species seem to be transported across the intestinal epithelium without harming the epithelial cells. These organisms then enter the lymphatic system and become distributed throughout many tissues in the host (43).

Like salmonellae, *Yersinia pseudotuberculosis* invades epithelial cells by an endocytic process which leaves endocytic vacuoles intact (1), and these organisms are also often transported into the mesenteric lymph nodes. Since *Y. pseudotuberculosis* is not actively motile at 37°C, chemotactic activity is apparently not involved in adherence or invasion in vivo. Outer membrane proteins mediate the adherence of *Y. pseudotuberculosis* to HeLa cells (2), and an outer membrane protein which mediates the invasion of these cell has also been recently identified (20). Additional studies with *Y. enterocolitica* have indicated that a plasmid-encoded cytotoxin is produced by internalized organisms, but such cytotoxic effects have not been reported in cells infected with *Y. pseudotuberculosis*.

### Diarrheal Stage

When ingested by rhesus monkeys, *S. typhimurium* invades mucosa of the jejunum, ileum, and colon. This infection is accompanied by the net secretion of fluids in all three portions of the intestine (35). The physiological basis of this transport abnormally may involve the activation of adenlyate cyclase, which is mediated by a choleralike enterotoxin produced by *S. typhimurium* (33). However, it is interesting that some fully invasive strains of *S. typhimurium* do not elicit significant mucosal inflammation, and these strains do not cause diarrhea (10). Therefore, it has been suggested that prostaglandins synthesized as a result of the acute inflammation associated with *Salmonella* enteritis may also activate mucosal adenylate cyclase (10). A role for a Shiga-like cytotoxin in eliciting this mucosal inflammation has been postulated (25). Arguing against this hypothesis, however, is the finding that HeLa cells infected with a toxin-producing strain of *S. typhimurium* continue to synthesize protein at a normal rate (13, 25). In addition to the determinants of the invasive phenotype, it is obvious that other virulence determinants are necessary for *Salmonella* enteritis, but the contribution of *Salmonella* toxins to this disease process remains a matter for conjecture.

## GENETICS OF VIRULENCE IN *SALMONELLA* AND *YERSINIA* SPP.

### *Salmonella* spp.

In contrast to localized *Shigella* infections, the disease process in salmonellosis involves invasion of the intestinal epithelium, spread of organisms to the mesenteric lymph nodes, and systemic dissemination. The genetic basis of this complex pathogenesis is not well understood. Nontyphoid *Salmonella* serotypes uniformly harbor large plasmids which are necessary for virulence in animal models (18), but the virulence determinant(s) encoded by the plasmids has not been characterized. Although it has been suggested that a 60-MDa plasmid is necessary for invasion of epithelial cells by *S. typhimurium* (22), current reports indicate that this plasmid is probably more closely associated with serum resistance and survival within macrophages (12, 18). *Salmonella dublin* also harbors a large plasmid (80 MDa), which is necessary for fatal systemic infections in mice, but is not involved in the initiation of infection at the level of the gut (D. Guiney, J. Fierer, G. Chikami, and P. Beninger, *Abstr. UCLA Symp. Mol. Cell. Biol., J. Cell. Biochem.* 11B:109, 1987). These observations suggest that the invasive phenotype is encoded by chromosomal genes in *Salmonella* species, but, like the genes encoding enterotoxins and cytotoxins, this marker has not been mapped.

### *Yersinia* spp.

The gene encoding the invasive phenotype in *Y. pseudotuberculosis* is also located on the chromosome, but unlike the invasion genes of *Salmonella* spp., this gene has been cloned into *E. coli* and its protein product has been char-

TABLE 1
Summary of pathogenesis and virulence determinants of enteroinvasive pathogens

| Organism | Target organ | Chemotactic motility | Adhesion | Endocytic protein | Intracellular fate | Additional virulence determinants |
|---|---|---|---|---|---|---|
| *Shigella* spp. | Colon | None | Plasmid-encoded, OMP(s)[a] | Plasmid-encoded *ipa* gene products | Escape from vacuoles, bacterial growth, and host cell death | Shiga and Shiga-like toxin, plasmid-encoded contact hemolysin |
| *Salmonella* spp. | Small intestine and colon | Yes | Chromosomal hemagglutinins | Unknown | Limited growth within vacuoles and translocation to basement membrane | Cholera-like enterotoxin, Shiga-like toxin, and plasmid-encoded survival mechanism for serum and RES[b] |
| *Yersinia* spp. | Small intestine | None at 37°C | Chromosomal OMP | Invasin | Same as for *Salmonella* spp. | Cytotoxin in *Y. enterocolitica* |

[a]OMP, Outer membrane protein.
[b]RES, Reticuloendothelial system.

acterized (20). The ability to invade tissue culture cells is conferred by a 3.2-kDa chromosomal region carrying a gene designated *inv*, and this gene expresses a 108-kDa outer membrane protein, which has been designated invasin. When bound to a solid matrix, invasin facilitates the adherence of HEp-2 cells. Therefore, it has been proposed that this protein causes *Y. pseudotuberculosis* to bind to the surface of epithelial cells in a way that induces endocytic uptake (R. R. Isberg, *Abstr. UCLA Symp. Mol. Cell. Biol., J. Cell. Biochem.* 11B:108, 1987). Since site-directed mutagenesis of the *inv* gene causes the loss of virulence in the orally challenged mouse, it would appear that invasin probably plays a role in the initation of intestinal infections in vivo (R. R. Isberg, submitted for publication).

## SUMMARY OF PATHOGENIC MECHANISMS OF ENTEROINVASIVE ORGANISMS

In the previous sections we have attempted to give a survey and synthesis of recent experimental observations as they relate to the pathogenesis of invasive enteric disease. Table 1 summarizes some of the salient points of comparison between the genera *Shigella*, *Salmonella*, and *Yersinia*. It should be remembered, however, that each of these genera elicits a range of clinical manifestations and that their underlying pathogenic mechanisms vary with both the host and the tissue. Nonetheless, we hope that the basic characteristics of these infections have been accurately recounted and that future research with the genetic tools which are revolutionizing the study of bacterial pathogenesis will allow the characterization of these disease processes on a molecular level.

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