[Frontiers in Bioscience 1, d131-145, 1 August 1996]

NEW INSIGHTS ON MOLECULAR PATHWAYS UTILIZED BY SALMONELLA SPECIES IN CELL BINDING

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1. ABSTRACT

Salmonella infections are a principal source of gastroenteritis and enteric fever in a variety of animals, including humans. An essential step in the development of Salmonella pathogenesis is the entry of bacteria into non-phagocytic cells, including those that line the intestinal epithelium. As a consequence of specific cues from the host intestinal micro-environment, Salmonella entry into the intestinal epithelium is the product of a multistep process that culminates in host cell membrane ruffling, and subsequent bacterial uptake. The events that trigger the internalization event appear to require an array of bacterial secreted proteins, exemplified by the formation of bacterial surface appendages (invasomes) which are important for the induction of host-cell signal transduction pathways that lead to membrane ruffling. In addition, during intestinal disease states induced by *Salmonella typhimurium*, transepithelial migration of neutrophils rapidly follows attachment of the bacteria to the epithelial membrane. Current evidence indicates that the intestinal epithelium plays a key role in orchestrating the inflammatory response to surface attached *S. typhimurium*. In this review, we explore current insights on the molecular pathways utilized by *Salmonella spp.* in cell binding that are important not only in the processes of Salmonella internalization but also in the generation of signals which lead to active states of intestinal inflammation.

Received: 6/19/96; Accepted: 7/9/96

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2. INTRODUCTION

Salmonella typhimurium is the most common serotype isolated from humans suffering from infectious gastroenteritis and correspondingly has long been recognized as a public health problem. Contact between the epithelial cell apical membrane elicits a variety of epithelial responses. Such epithelial responses are likely triggered by specific contact-dependent, bacterial derived signals which are themselves modulated by physical characteristics of the microenvironment such as oxygen tension and osmolarity (1-5). Subsequent to such alterations, Salmonella may be internalized in a membrane bound vacuole and may translocate across the intestinal epithelium (6-7). The details of how such Salmonella-intestinal epithelial contacts evoke the classical histogical lesion of neutrophil transepithelial migration are incomplete (7-10). However, it is clear that transepithelial migration of neutrophils occurs early after Salmonella and epithelial contact (7), and well before the epithelium loses its structural

integrity (8). Such observations, however, imply that contact between the bacterial outer membrane and the epithelial apical membrane results in the generation of signals which directs the subsequent trafficking of neutrophils. Thus, current paradigm suggests that the host cell plays an active role in both Salmonella internalization, as well as in the orchestration of inflammatory responses. What follows is a brief review of the molecular pathways used by Salmonella in epithelial cell binding which govern the mechanisms of Salmonella internalization and the promotion of intestinal inflammation.

3. THE NATURE OF SALMONELLA-INTESTINAL INTERACTIONS

3.1 Salmonella and environmental cues

Salmonella spp. are facultative intracellular pathogens that cause a variety of diseases in both humans and animals, which range from a self-limiting enterocolitis (food poisoning) to more systemic illnesses such as typhoid fever. The type of diseases caused by these organisms depends not only on the serovar or species of the infecting bacteria but also on the species of the infected host. Some serotypes such as *S. typhi* are host adapted, in this case for humans, while others, such as *S. typhimurium* and *S. enteritidis*, can cause disease in a large variety of hosts (11). *S. typhimurium*, for example, will specifically cause self-limited gastroenteritis in immuno-competant humans, while in mice it will cause a severe systemic illness, much like typhoid fever. *Salmonella* infection is initiated when bacteria enter a host via contaminated food or water. Following passage through the stomach, the organisms move into the gastrointestinal tract of the host, and upon reaching the distal ileum, establish contact with a cellular target within the intestinal mucosa. As a result of such associations *Salmonella* are able to initiate passage through the intestinal epithelium where they can gain access to the reticuloendothelial system, thus providing an avenue for the dissemination to the lymph nodes, spleen, liver, and blood (12).

Once in the gastrointestinal microenvironment, the microorganism may interact with the apical membranes of columnar intestinal epithelia (7) or with specialized cells, termed M cells, which lie over the Peyer's patches (7, 13-14). The relative contributions of these interactions to the pathogenesis of disease is uncertain. In mice, evidence suggests that early entry of *S. typhimurium* appears to be via transepithelial transport by M cells to the Peyer's patches (12-13). M cells represent a minor constituent of the epithelial surface (far less than 1%) (15), and are specialized epithelial cells that appear to be designed for taking up large particles and, in addition, are believed to be important in antigen sampling. Some pathogens which are able to associate with and translocate across columnar intestinal epithelia use the M cell pathway to enter the host (for instance reovirus) (16). Moreover, enterocytes can also be invaded by *S. typhimurium*, providing an additional portal of entry (7). Columnar epithelial cells of the intestine constitute the major portion of surface area (15), are known to bind *Salmonella* and internalize it, and are the site at which neutrophil transmigration in response to such surface colonization is known to occur (7-10). Thus, it appears that while a number of microorganisms are able to enter the host through the M cells, invasion of enterocytes seems to be a less restricted more prevalent route. Although M cell and *Salmonella* associations play a role in host immunity to this organism, the bulk of procaryotic/eucaryotic interactions in primary colonization of the intestine by *Salmonella* likely occurs over the general columnar epithelial surface as suggested by studies of Takeuchi (7).

Once within the gastrointestinal microenvironment, the bacterium is exposed to extremes of temperature and pH, oxygen tension, bile salts, digestive enzymes, and a multitude of diverse, competing microorganisms. Such distinct, and seemingly hostile environments, are not only tolerated by the bacteria but importantly, serve as environmental signals for the microbe to initiate transcription of genes specifically adapted for host-microbe interactions. Thus, it is not suprising that the expression of *S. typhimurium* virulence factors important to interactions with epithelia is influenced by various environmental stimuli including oxygen (17), osmolarity (2, 18), and growth phase (1, 3, 18); conditions known to have effects on the level of DNA superhelicity (2). For example, previous reports have indicated that *Salmonella* adherence to and subsequent invasion into cultured epithelial cells was greatest either during the late logarithmic phase of growth, presumably due to oxygen limitation, or when the bacteria were grown either anaerobically or incubated with cells under anaerobic conditions (1, 3). Furthermore, an assay in which a short bacterium-cell interaction period was used, also concluded that only *Salmonella* grown under low oxygen conditions, but not bacteria from stationary phase cultures, elicited rapid changes in cell morphology, internal actin filament rearrangement, and cell entry(17). Recent work (5), however, has subsequently demonstrated that invasion of *S. typhimurium* by epithelial cells could be reduced during utilization of carbohydrates and that the repression of cell association by certain carbohydrates (*i.e*. glucose) was greater during aerobic growth of the bacteria. Thus, this study suggests that previous reports of greater cell invasion by *S. typhimurium* during anaerobic growth may have risen from the use of media

containing carbohydrates which were found to be more repressive during aerobic growth of the bacterium (5). Nonetheless, depletion of a preferred carbohydrate substrate in the presence of other potential carbon energy sources represents a nutrient limitation that can stimulate a responsive change and adds to the record of environmental stimuli that control the capability of *Salmonellae* to associate with and invade epithelial cells *in vitro* (5).

S. typhimurium virulence can also be controlled, at least in part, by the global regulatory network, PhoP/PhoQ (19-22). Such two component regulators are members of a family of environmental sensors (PhoQ) and transcriptional activators (PhoP) that are required for the expression of genes termed *pag* (*phoP*-activated-genes) (23-25). *pag* are transcriptionally activated within acidified macrophage phagosomes several hours after phagocytosis and are required for intracellular survival (20, 24, 26). In addition to the ability to transcriptionally activate *pag*, PhoP/PhoQ can repress the synthesis of proteins encoded by genes designated *prg* (*phoP* repressed-genes) (19 21). *prg* products are likely to play important roles in *S. typhimurium* signaling to eucaryotic cells which include; (i) induction of macrophage generalized membrane ruffling, macro-pinocytosis and internalization of bacteria within spacious phagosomes (27-28); (ii) induction of bacterial mediated endocytosis (BME) by epithelial cells (11); and (iii) induction of polymorphonuclear leukocyte transmigration across polarized epithelial cell monolayers (29) (see below). Recent evidence also indicates that Mg^{2+} is an extracellular environmental signal that controls the PhoP/PhoQ regulon (30).

3.2 Salmonella pathogenesis and host cell invasion

The ability to penetrate the cells of the intestinal epithelium is an essential step in the pathogenic cycle of the enteric pathogen *Salmonella* (7). The *Salmonella* invasion process is thought to involve the interaction of determinants on the surface of the bacteria with the host cell, triggering an event that resembles macropinocytosis, referred to as bacterial mediated endocytosis. Observations by Takeuchi (7) first provided the groundwork for understanding the sequence of events that leads to the entry of *Salmonella* into intestinal epithelial cells. Via electron microscopic studies, he was able to demonstrated that the microvilli of the intestinal epithelium underwent dramatic changes after *Salmonella* came into close proximity to the brush border. Such changes, exemplified by membrane ruffles, localized to the point of bacterial contact and were transient, since after internalization of the bacterium the microvilli recovered their normal, preinfected appearance. Neither the molecular basis for this phenomenon, which appears to be unique to *Salmonella* and *Shigella* , nor the significance of this event, are completely understood. Our current understanding is that the ability of *Salmonella* to induce formation of membrane ruffles is critical for entry since mutants unable to induce these changes are severely impeded in their ability to enter cultured mammalian cells (31, 32). *Salmonella* are then subsequently internalized via membrane bound vacuoles formed from such membrane ruffles. This process is termed macropinocytosis, and results in the formation of spacious phagosomes (26-28, 33-34). The appearance of membrane ruffles on the surface is accompanied by profound cytoskeletal rearrangements at the point of bacterial- host cell contact, which require a number of cytoskeletal proteins, including actin, alpha-actinin, talin, tubulin, tropomysin, and ezrin (35). Although the significance of the recruitment of several of these proteins is unclear, actin is likely to play a role in the formation of membrane ruffles since inhibitors of actin microfilament function blocks *Salmonella* entry (36-37). Other organisms have also been shown to induce cytoskeletal alterations. *Listeria monocytogenes*, for example, continues to generate interest by virtue of its ability to induce locomotion through directional actin assembly within the host cells (38-41). Previous results have demonstrated that actin-rich rocket tails trailing behind motile bacteria became anchored in the cytoplasm (38, 42-43). Such rocket tails create physically confining boundaries that control the direction of bacterial movement. *Yersinia* spp. can also influence host cytoskeletal proteins. Entry of enteropathogenic *Yersinia* into cultured mammalian cells has been described as parasite-specified phagocytosis (44), in which movement of the host cytoskeleton in response to signals is sent from a transmembrane receptor that is recognized by bacterially encoded ligands. For example, previous results have indicated that actin, and actin associated proteins, such as filamin and talin, accumulate around the entering bacterium (45). *Yersinia* invasin is responsible for such activity, and this observation is consistent with the finding that invasin recognizes multiple beta 1 integrins (46).

S. typhimurium infection of cultured epithelial cells is also accompanied by a marked increase in $[Ca^{2+}]i$ flux (32, 47), an event which is most likely necessary for internalization and may play a role in the formation of membrane ruffles. Other cellular responses include tyrosine phosphorylation of a number of host proteins along with the epidermal growth factor receptor and the initiation of signal transduction pathways which ultimately lead to the activation of phospholipase A2, and production of arachidonate metabolites (47). However, Francis et al (28) found that invasive *Salmonella* elicited characteristic ruffles in Swiss 3T3 fibroblast and NR-6 cells, a 3T3 derivative that does not express the

EGF receptor. Moreover, invasive *Salmonella* also elicited membrane ruffling in rat basophilic leukemia cells, despite failure of EGF to elicit ruffling in these cells (28). Thus, this study suggests that the EGFR is not required for *S. typhimurium* invasion, although there may be alternative (parallel) pathways that cause either direct or indirect phosphorylation of the EGF receptor during *Salmonella*-induced ruffling. Nonetheless, it is interesting to note that the events of membrane ruffling, cytoskeletal rearrangements, and Ca^{2+} influxes also occur as part of a global cellular response to mitogens, oncogene expression, and growth factors (48-50) and are associated with enhanced pinocytosis (48). Thus, the formation of membrane ruffles comprises the macropinocytotic machinery mediating pinocytosis, and is subverted by *Salmonella* so as to enter into diverse mammalian cells, perhaps reflecting the existence of a common pathway for ruffle induction.

3.3 Salmonella entry into epithelial cells is determined by genetically defined factors

Animal cells in culture as well as transformed human cell lines have become popular *in vitro* models for studying attachment to and invasion into epithelial cells (6, 51-58). Although, genetic approaches have been used to identify the *Salmonella* factors that directly interact with the epithelial cells and facilitate invasion, such studies have indicated that the genetics controlling these processes are complex and involve multiple chromosomal loci (59-62). For example, *S. typhi* genes have been identified which, when cloned into *Escherichia coli* K-12, allow this normally non-invasive bacterium to enter cultured cells (63). In addition, Stone *et al.*, (62) has identified a number of Tn*phoA* mutants of *S. enteritidis* that render these organisms defective for invasion into cultured epithelial cells. Such mutants mapped to 9 different loci on the chromosome and affected entry to different degrees. Using a similar technique, Betts and Finlay (59) isolated transposon mutants from *S. typhimurium* in 4 distinct loci that also rendered these organisms deficient for entry into epithelial cells. Together these studies imply that *Salmonella* may encode alternative entry pathways since it has not been determined whether these different loci are functionally related. Moreover Lee *et al.*, developed a strategy which selected for mutants of *S. typhimurium* which were competent for cell entry into epithelial cells only under non-physiologic conditions (*i.e.* during non microaerophilic growth) (61). Such a strategy identified the *hil* locus which is essential to bacterial entry into cultured epithelial cells, and which presumably encodes a regulatory factor required for proper expression of entry determinants. The PhoP-repressed locus *prgH* was also previously identified as being important for signaling epithelial cells to endocytose *S. typhimurium*. Characterization of *prgH* revealed that it is an operon of four genes *prgHIJK*, strongly linked to the *hil* locus (19). Synthesis of the transcript was repressed in bacteria that activate PhoP/PhoQ, thus indicating that PhoP/PhoQ regulates *prgHIJK* by transcriptional repression. Another successful strategy for the detection of *Salmonella* invasion genes, was developed by Galan and Curtiss who identified a *S. typhimurium* genetic locus, *inv*, based on its ability to complement a non-invasive strain of *S. typhimurium* (60). Subsequent analysis of this locus has identified at least 14 genes, which upon mutation affected the ability of *Salmonella* spp. to enter cultured epithelial cells, without affecting the ability of these cells to attach, suggesting that attachment and entry are genetically separate events in *Salmonella*. These genes are apparently arranged in the same transcriptional unit and were mapped to 59 min on the *Salmonella* chromosome (31) near the *hil* (64) and *prg*HIJK loci (19). Subsequent to the discovery of *inv*, a new assemblage of genes responsible for invasion properties of *Salmonella* were identified which were remarkably similar in order, arrangement and sequence to the gene cluster controlling the presentation of surface antigens (spa) on the virulence plasmid of *Shigella* (46). In *Salmonella*, this chromosomally encoded complex, also called *spa*, consists of over 12 overlapping or adjoining genes with the *inv* locus, suggesting a single transcription unit (*inv/spa* complex) (65).

To date some 25 invasion genes, as shown in Figure 1, have been found to be clustered near minute 63 of the *S. typhimurium* chromosome (19, 31, 61-63, 65), and 40 kb of unique DNA may be necessary for entry of *Salmonella* into mammalian cells. Interestingly, the finding that a non-invasive *spa* mutant of *Salmonella* could be rescued by the corresponding *Shigella* homologue, provided the initial evidence that *spa*, perhaps, promotes equivalent functions in *Shigella* and *Salmonella* (Figure 1). Presumably, this gene cluster has been acquired independently by each genus yet displays motifs used by diverse antigen export systems including those required for flagellar assembly and protein secretion (65). For example, a number of predicted *inv* gene products have been identified and are similar to proteins thought to be involved in export and assembly of bacterial flagellar components (65-68). Among these homologues are proteins involved in flagellar assembly in *E. coli* (FlhA, Flil, FliJ, and FliN), *Bacillus subtilis* (FliP and *flaA* locus), as well as in bacteriophage assembly (Protein IV and Pf3). Furthermore, recent observations have indicated that 12 of these genes, *invG*, *invE*, *invA*, *invB*, *invC/spaL*, *spaM*, *spaN*, *spaO*, *spaP*, *spaQ*, *spaR*, and *spaS*, have the identical gene order and significant sequence similarity to the *Shigella mxi* and *spa* genes (31, 65-70). The *mxi* and *spa* genes are

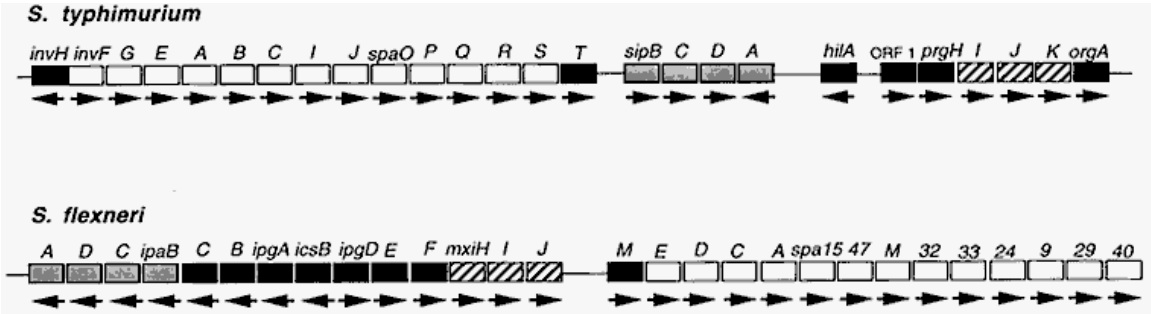

Figure 1. Similarities between the genetic organization of the invasion genes clusters from *Salmonella typhimurium* and *Shigella flexneri*. This map shows the relative positions and the transcriptional directions of the genes illustrated, as indicated by the position of the arrow. Gene clusters which are conserved both in sequence and in gene order are indicated by the following key: *inv*/*spa*;*mxi*/*spa* (open/white bars), *prgIJK*;*mxiHIJ* (diagonal stripped bars), and *ipa*;*sip* (stippling). Black bars indicate genes with no homologues within the respective regions. The map organization was compiled from information published by the following investigators (65, 69, 74, 84-85, 88).

encoded on the large *Shigella* virulence plasmid and are required for export of Ipa proteins (invasion protein antigens) which facilitate *Shigella* entry into mammalian cells (68-69, 71-73). Additionally, the role of the *prgHIJK* operon in BME was supported further by the finding that some of its predicted gene products of this locus were similar to *S. flexneri* secretion determinants that are essential for epithelial cell invasion. For example, the *prgI*, *prgJ*, and *prgK* predicted gene products of *S. typhimurium* were recently found to be similar to the MxiH, and MxiI, and MxiJ proteins, respectively, of *S. flexneri* (74). The relationship of such genetic organization of the invasion genes between *S. typhimurium* and *S. flexneri* is depicted in Figure 1. Such Salmonellae genes also show sequence similarity to several genes that encode proteins involved in the surface presentation and/or secretion of a variety of molecules in a number of other organisms including, *Yersinia* (LcrD, LcrE, and YscA), *Klebsiella* (PulD), *Aeromonas hydrophila* (ExeD), and *Xanthomonas campestris* (PefD). The significance of these findings is that these homologies indicate that *Salmonella* may externalize invasion proteins by a mechanism that is functionally similar to that involved in flagellar export and assembly, such that *Salmonella*, like *Yersinia*, may assemble a supramolecular structure on its surface, in order to induce its internalization into mammalian cells.

3.4 Salmonella and secreted invasion determinants

To this end, in studies recently performed by Miller and colleagues (75), analysis of the culture supernatants from wild-type *S. typhimurium* demonstrated that at least 25 polypeptides larger than 14 kDa were detected. In contrast, *prg*H1:Tn*phoA*, phoP-constitutive, and *hil* deletion mutants had significant defects in their supernatant protein profiles. These results suggest that PhoP/PhoQ regulates extracellular transport of proteins by transcriptional repression of secretory determinants and that secreted proteins may be involved in signaling the epithelial cells to endocytose bacteria (69). Because of the similarity between predicted gene products from the *prgHIJK* operon and gene products required for protein secretion in other bacterial species, an analysis of proteins present in culture supernatants of *S. typhimurium* was performed (75). This analysis suggested that PhoP/PhoQ could control protein secretion, at least in part, by repressing *prgHIJK*, whose product could form part of a secretion machinery. Since the strains with altered Ssp profiles were impaired in signaling epithelial cells, this report suggests that Ssp are involved in signaling such cells to initiate BME. The possibility that *Salmonella* proteins form an apparatus assembled on the cell surface that is necessary in order to signal eucaryotic cells was suggested by the work of Galan and colleagues (67), who demonstrated that *S. typhimurium* forms a novel surface structure which is lost as the organism enters membrane ruffles of epithelial cells. The release of this apparatus from the cell surface during growth in culture could result in the detection of these proteins in the supernatant. Specifically, contact between *S. typhimurium* and epithelial cells resulted in the formation of appendages (invasomes) on the surface of the bacteria, which did not require *de novo* protein synthesis, and was a transient event (67). Such, appendages were immediately shed or retracted before or subsequent to signaling the host, since *S. typhimurium* associated with membrane ruffles did not exhibit these surface structures. Moreover, such surface structures were not seen on organisms unexposed to the host cells, and *S. typhimurium* mutants defective in the transient formation of these surface appendages were unable to enter into cultured epithelial cells, suggesting that these structures are required for bacterial internalization. As a consequence of this interaction, appendages are

specifically assembled on the surface of the Salmonella, a process which appears to be required for subsequent triggering of the host cell signal-transduction pathways that lead to membrane ruffling and the internalization of these organisms (31, 47, 67). Thus, such intimate interactions represent a phenomenal example of reciprocal biochemical signaling between a pathogen and the host, itself, and emphasizes the notion that *Salmonella* can sense environmental signals from the host cell resulting in the transient assembly of a surface organelle on the bacterium.

3.5 Salmonella and the protein secretion apparatus

Salmonella entry (internalization) into host cells has recently been found to require the function of a dedicated, sec-independent, type III protein secretion system encoded in the *inv* and *spa* loci located at minute 59 on the *Salmonella* chromosome (76). Presumably, such a translocation apparatus would actively participate in the host cell contact-dependent assembly of a supramolecular structure, presumably required for the presentation and/or delivery of invasion determinants to the target cell. Evidence to support this notion is exemplified by the fact that invasome assembly, itself, requires a functioning type III secretion system. The type III secretion systems are usually encoded by genes that are clustered together on the chromosome (*Salmonella* ssp.(76)) or on large plasmids (*Shigella*, *Yersinia* (77, 78)). One protein common to all of the type III systems is an ATPase which presumably energizes the transport system. In *Salmonella* spp. this is InvC, which is homologous to *spa47* from *Shigella* (73) and *yscN* of *Yersinia* spp. (79-80). Another common component of the type III systems is an outer membrane-associated translocase that is homologous to PulD from *K. oxytoca* (81). This protein is InvG in *Salmonella* spp. (70), MxiD in *Shigella* spp. (69) and YscC in *Yersinia* spp. (82). Similar type III secretion systems are also required for the virulence phenotype of other pathogenic bacteria including *Yersinia* spp., *Shigella* spp., and enteropathogenic *E. coli*, as well as a number of plant pathogens from the *Xanthomonas*, *Pseudomonas*, *Aeromonas*, and *Erwinia* genera (83-84). Such a secretion system is distinct from both the type I (sec-independent) protein secretion system exemplified by the export of the *E. coli* heamolysin, and the type II (sec-dependent) general secretory pathway of gram negative bacteria exemplified by the secretion of pullulanase of *Klebsiella oxytoca*.

Several proteins have currently been identified whose secretion into the culture supernatant of *S. typhimurium* is dependent on the type III secretion system (85), and in addition have determined to be potential components of the invasome structure (67). InvJ was the first identified target of the protein secretion apparatus, and exhibited significant sequence similarity to the EaeB protein of enteropathogenic *E. coli*. Initial observations found that 30% of the product of *invJ*(*spaN*) was recovered in the culture supernatant of wild-type *S. typhimurium* but not from that of *invG* or *invC* mutants (86). Moreover, mutations in *invC* or *invG* prevented the assembly of the invasome and dramatically reduced *Salmonella* entry into the host cells, indicating that there is a close correlation between invasome assembly and the internalization process. Although the function of InvJ is yet to be determined, it is thought to be a candidate for either a structural component of the invasome appendage, or alternatively, InvJ secretion may precede invasome formation, serving as a signal to assemble the appendage (87). Other genes encoding secretion proteins have been identified and include *sipB*, *sipC*, *sipD*, and *sipA* (85-86). Such genes encode polypeptides that have significant sequence homology to the IpaB, IpaC, and IpaA proteins of *Shigella* spp., respectively (85-86, 88), and are themselves targets of the type III secretion system (Figure 1).

4. INFLAMMATORY RESPONSES ELICITED BY SALMONELLA-INTESTINAL EPITHELIAL INTERACTIONS

4.1 Salmonella coordinates mucosal inflammatory responses

Despite progress made in understanding the mechanisms of secretory diarrhea produced by bacterial toxins, such as cholera toxin, how bacterial pathogens such as salmonellae cause gastroenteritis is poorly understood. It has long been recognized that attachment of non-typhoidal *Salmonella* serotypes such as *S. typhimurium* to the intestinal epithelium provoke an intense intestinal inflammatory response, consisting largely of neutrophil (polymorphonuclear leukocyte (PMN)) migration across the epithelial lining of the intestine (7). This inflammatory event manifests itself as epithelial dysfunction, namely, diarrhea (7-10, 64, 89). The details of how such *S. typhimurium*-intestinal epithelial contacts evoke the classical histological legion of neutrophil transepithelial migration are not well characterized (7, 9-10). However, while it is clear that transepithelial migration of neutrophils occurs early after *Salmonella*-epithelial contact (7), and well after the epithelium loses its structural integrity (8), the mechanisms and cell types responsible for coordinating mucosal inflammatory responses to such pathogens remain largely obscure. Evidence is emerging, however, that bacterial binding to eucaryotic cells can influence the program of transcriptional regulation for synthesis of biologically important eucaryotic products. For example, Interleukin-6 (IL-6) production is stimulated by the binding of adherent *E. coli* to bladder or kidney

epithelial cells (90), and LPS (lipopolysacchride) has been shown to stimulate tumor necrosis factor (TNF), Interleukin-1 (IL-1), IL-6, and Interleukin-8 (IL-8) production in a host of cell types including monocytes, fibroblasts, and endothelial cells (91-93). IL-8 is of particular importance since unlike the other cytokines listed, it is a potent PMN chemotaxin when present in a gradient (94). Moreover, a recent report (95) modeling urinary tract infections (UTIs) also provided direct evidence that urinary epithelia cells exposed to *E. coli* secrete IL-8, and such observations fit well with studies indicating that IL-8 can be recovered in the urine of patients suffering with UTI. Taken together, such observations imply that contact between the bacterial outer membrane and the cell apical membrane results in the generation of a signal(s) which may be important for the initiation and amplification of the mucosal inflammatory response.

4.2 Salmonella contact with the intestinal epithelium generates signals important for the initiation and amplification of the mucosal inflammatory response

How might such transepithelial signaling of underlying inflammatory responses occur? Recent studies show that *S. typhimurium* contact with the apical pole of intestinal epithelial cells generates signal(s) which may be responsible for directing the trafficking of neutrophils across the intestinal epithelium (29, 96-99). The transepithelial migration of neutrophils in response to luminal pathogens necessarily involves movement through several anatomic compartments, each with their own complexities: (a) the well recognized rolling, firm adhesion, and subsequent emigration of neutrophils from the microvasculature (100-107); (b) subsequent migration of neutrophils across the lamina propria and into a subepithelial position; and (c) transepithelial migration. While the mechanisms driving these responses have only recently attracted attention, it is clear that bacterial binding to epithelial cells can influence the production of important regulators of inflammation. For example, McCormick *et al*. (99), has previously demonstrated using *in vitro* models of intestinal inflammation, that apical attachment of *S. typhimurium* to intestinal epithelial monolayers specifically stimulates physiologically directed neutrophil transepithelial migration. The signals responsible for orchestration of this response do not utilize the neutrophil n-formyl peptide receptor directed migration - the best understood receptor-mediated pathway from directing neutrophils to a bacterial target (99). However, among the events stimulated by such pathogen and host interactions is the release of chemotaxins which might guide neutrophils to the site of bacterial-epithelial contact (96-99). For example, *S. typhimurium*-intestinal epithelial cell interactions induce the epithelial synthesis and basolateral release of the potent neutrophil chemotactic peptide IL-8 (96, 98-99). The mechanisms responsible for such basolateral IL-8 secretion are not well characterized. Eckmann *et al*., (96), however, has suggested that IL-8 secretion elicited by *S. typhimurium* contact with the epithelial cells may require signaling associated with cell entry. In contrast, recent evidence indicates that such IL-8 stimulated induction by *Salmonella* may be more complex. For example, current evidence indicates *Salmonella* strains or serotype-related differences in the ability of salmonellae to induce diffuse enteritis in human does not correlate well with the ability of these organisms to be internalized by intestinal epithelial cells. Yet, the ability of *Salmonella* interactions with the apical pole of intestinal epithelial cells to elicit transepithelial signaling to neutrophils correlates well with *Salmonella* serotyes which elicit diffuse enteritis in humans (29). Thus, such evidence strongly suggests that the ability of Salmonellae to elicit transepithelial signaling to neutrophils is a key virulence mechanism underlying *Salmonella*-elicited enteritis.

Based on these observations, it is becoming increasing clear that interactions between intestinal epithelial cells and *S. typhimurium* may play a key role in orchestrating the inflammatory response. This is further exemplified by studies performed by Jung *et al.* (97) who demonstrate that in response to bacterial invasion of a variety of human colon epithelial cell lines, a specific array of four proinflammatory cytokines (IL-8, monocyte chemotactic protein-1(MCP-1), TNF alpha, and GM-CSF), was found to be coordinately expressed and upregulated in human colon epithelial cell ines (97). The coordinate expression of these proinflammatory cytokines seems to be a general property of human colon epithelial cells since freshly isolated human colon epithelial cells had identical responses (55), and suggests that such cytokine production may play an essential role in intercellular communication by delivering signals which influence the target cells upon which they act.

While each of these cytokines plays a critical role in the initiation and amplification of the inflammatory response, only IL-8 acts as a potent neutrophil chemoattractant. Thus, since neutrophils must initially emigrate from the microvasculature to the subepithelial compartment (47, 104, 107) it was originally hypothesized (22, 99) that a potential role for such basolateral IL-8 secretion may be in the recruitment of neutrophils through the epithelial matrix to the subepithelial space, rather than in directing the final movement of neutrophils across the intestinal epithelium. One unique aspect of the

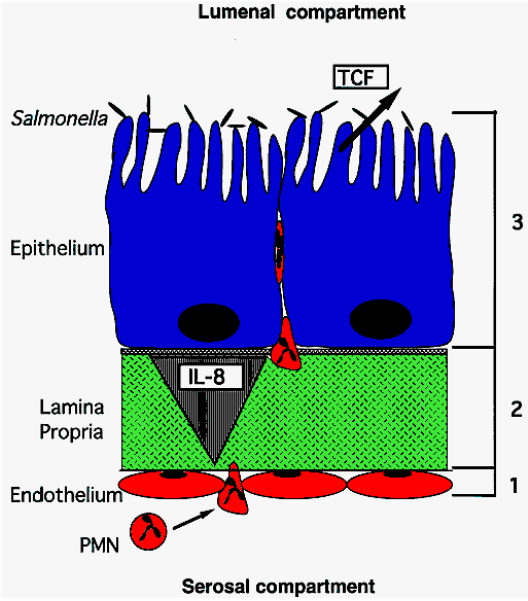

Figure 2. Multistep model of transepithelial of neutrophils across the intestinal mucosa in response to apically attached *S. typhimurium*. Such neutrophil transmigration in response to luminal pathogens involves movement through several anatomic compartments, each with their own complexities. Step 1: Initially, neutrophils must migrate from the microvasculature to the subepithelial lamina propria. This process is best understood in terms of the molecular interactions that correspond to the initial tethering (selectin mediated) and subsequent firm attachment (β2 integrin mediated) of neutrophils to the endothelial surface. Set 2: IL-8 is released from the basolateral aspect of epithelial cells in response to adherent *S. typhimurium.* IL-8 imprints the subepithelial matrix with retained haptotactic gradients sufficient to resist washout effects of the volume that normally traverses this compartment. Thus, the primary role for basolateral secretion of IL-8 is the recruitment of neutrophils through the matrix to the subepithelial space, rather than directing the final movement of neutrophils across the epithelium. Step 3: Recent evidence indicates that an apically secreted soluble factor with physical characteristics unlike IL-8 (B. A. McCormick and J. L. Madara), acts in concert with IL-8, and appears responsible for driving this final in neutrophil transepithelial migration (modified from reference 99).

intestinal mucosa which might require tandem signaling events for this process is the presence of a vascular countercurrent arrangement in the subepithelial compartment (108). As happens for absorbed solutes, this countercurrent phenomenon may distort transepithelial solute gradients. For example, perfusion of mammalian intestinal loops *in vivo* with solutions containing fMLP was previously found to induce neutrophil attachment to endothelial cells and structurally defined endothelial activation, but failed to elicit directed migration across the lamina propria (Madara, unpublished observations), suggesting that directed migration may require a more stable gradient than that afforded by the usual soluble signals. For example, once present in inflamed tissue, IL-8 is likely to retain its biological activity for several hours, as shown by local intradermal administration in animals and humans (109-111). In contrast to IL-8, chemokines such as fMLP or LTB4 are degraded rapidly by oxidation or hydrolysis (112). Thus, gradients of IL-8 formed across the lamina propria matrix would likely be relatively resistant to the distorting effects of the complex solvent flow patterns which exist in this microenvironment and could serve to bring PMN into the subepithelial space. Most importantly for sites like the subepithelial matrix of intestinal mucosa where volume flow is extremely high, IL-8, due to its highly cationic nature, binds avidly to glycosaminoglycans of the tissue matrix (113), thus making such bound IL-8 gradients particularly resistant to sweeping away affects of fluid flow.

Consistent with this notion, recent evidence indicates that biophysically confluent T84 cellmonolayers apically colonized by *S. typhimurium* resulted in the imprinting of a neutrophil chemotactic signal on the underlying epithelial-derived matrix which was primarily due to the basolateral secretion of the C-X-C- family member, IL-8 (98). Such studies provided evidence to substantiate the notion that basolateral IL-8 secretion may act to guide neutrophils through the matrix to the subepithelial space. For example, recent investigations (98) indicate that when underlying matrices were isolated from biophysically confluent polarized monolayers of the human intestinal epithelial cell line T84, they failed to support substantial transmatrix migration of PMN unless an exogenous chemotactic gradient was imposed. However, such matrices isolated from confluent monolayers apically colonized with *S. typhimurium*, supported spontaneous transmatrix migration of PMN. Such chemotactic imprinting of underlying matrices was resistant to volume wash and as also paralleled by secretion of the known matrix binding chemokine IL-8. Moreover, such chemotactic imprinting of the matrix underlying *S. Typhimurium* colonized monolayers was found to be independent on epithelial protein synthesis, was directional implying the existence of a basolateral-driven gradient, and was neutralized by antibodies either to IL-8 or to the IL-8 receptor on PMN. Even an avirulent *S. typhimurium* strain, $PhoP^{c}$, which attaches to epithelial cells as efficiently as wild-type *S. typhimurium*, but fails to induce basolateral secretion of IL-8, failed to imprint matrices. Together, these observations clearly indicate that the epithelial surface can respond to the presence of a lumenal

pathogen and subsequently imprint the subepithelial matrix with retained IL-8 gradients sufficient to resist washout effects of the volume flow which normally traverse this compartment. The impact of such events may have substantial importance in assisting movement of neutrophils to the subepithelial space. Additionally, it has been noted in patients with cystic fibrosis that neutrophil elastase can induce IL-8 gene expression in respiratory epithelia (114), and inhibition of IL-8 gene expression by aerosolized secretory leukoprotease inhibitor suppresses both IL-8 secretion and neutrophil infiltration of this epithelium in this disorder (115). Such data further support the notion that the primary role for basolateral secretion of IL-8 by the intestinal, and likely other epithelia, is recruitment of PMN through the matrix to the subepithelial space, rather than directing the final movement of PMN across the epithelium. It is now speculated that IL-8 may act in concert with a transcellular chemotactic factor which directs neutrophil migration across the intestinal epithelium (98). Figure 2 illustrates the current paradigm of neutrophil emigration in response to apical epithelial attachment of *S. typhimurium*. While there is now substantial evidence that the epithelial cells themselves may play a proactive role in organizing/initiating such inflammatory responses (95-97, 99), a recent precedent has also been established that indicates that products, other than n-formyl peptides, from enteric bacteria might play a more complex role in regulating the activity of the mucosal immune system. Products of *E. coli* were shown to regulate lymphocyte activation and cytokine production, and suggest that these products may have important influences in modifying gastrointestinal immune responses to enteric bacterial infection (116). Additionally, cell bound components of *Helicobacter pylori* (gastric inflammatory response) were shown to release factors that are chemotactic for neutrophils and/or monocytes (117-122). One study showed that the N-terminal end of the large subunit of *H. pylori* urease was chemotactic for neutrophils (120), and other, as yet identified, soluble chemotactic factors of *H. pylori* have also been described (119, 122-123).

5. PERSPECTIVE

In recent years some eminent advances have been made in the basic understanding of how Salmonella interact with intestinal epithelial cells, their host cell target. Specifically, this review examined the current insights on the molecular pathways utilized by *Salmonella spp.* in cell binding that are important for the promotion of *Salmonella* disease pathogenesis. The most progress has been made in the area of the molecular genetic basis of Salmonella entry into mammalian cells. However, there has recently been an emanation of information concerning the basic understanding of how Salmonella binding to epithelial cells coordinates the mucosal inflammatory response. Importantly, a common theme to emerge from such fertile areas of research is that upon Salmonella binding to epithelial cells, the host cell plays an active role in not only Salmonella internalization, but also in generating proinflammatory signals which lead to active states of intestinal inflammation. Of course, while many questions await answers, future investigations can only shed more light on the pathogenic mechanisms which govern Salmonella binding to epithelial cells.

6. ACKNOWLEDGMENTS

These studies were suppored by National Insitiute of Health grants DK50989 to B.A. McCormick, DK47662 to J.L. Madara, and AI30479 to S.I. Miller.

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