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THE ROYAL SOCIETY

The pathogenesis of *Shigella flexneri* infection: lessons from *in vitro and in vivo* studies

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Shigella flexneri is a Gram-negative facultatively intracellular pathogen responsible for bacillary dysentery in humans. More than one million deaths occur yearly due to infections with *Shigella* spp. and the victims are mostly children of the developing world. The pathogenesis of *Shigella* centres on the ability of this organism to invade the colonic epithelium where it induces severe mucosal inflammation. Much information that we have gained concerning the pathogenesis of *Shigella* has been derived from the study of *in vitro* models of infection. Using these techniques, a number of the molecular mechanisms by which *Shigella* invades epithelial cells and macrophages have been identified. *In vivo* models of shigellosis have been hampered since humans are the only natural hosts of *Shigella*. However, experimental infection of macaques as well as the murine lung and rabbit ligated ileal loop models have been important in defining some of the immune and inflammatory components of the disease. In particular, the murine lung model has shed light on the development of systemic and local immune protection against *Shigella* infection. It would be naive to believe that any one model of *Shigella* infection could adequately represent the complexity of the disease in humans, and more sophisticated *in vivo* models are now necessary. These models require the use of human cells and tissue, but at present such models remain in the developmental stage. Ultimately, however, it is with such studies that novel treatments and vaccine candidates for the treatment and prevention of shigellosis will be designed.

Keywords: *Shigella*; pathogenicity; mucosal immunity; immune response; animal models; *in vitro* models

1. INTRODUCTION

nfection with *Shigella* spp. is a serious cause of morbidity nd mortality especially in children of the developing orld. Recently, the World Health Organization esti- nated that 1.1 million deaths per year are attributed to higellosis (Kotloff *et al.* 1999). There are four species of *higella* that cause these infections, with *S. flexneri* and, to a esser extent, *S. sonnei*, accounting for most of the endemic isease. Epidemic disease is usually due to *S. dysenteriae*, hich displays the same invasive capacity as the other pecies but in addition, secretes a potent cytotoxin, Shiga oxin, that can cause haemolytic uraemic syndrome. Existing antimicrobial treatments are becoming increas- ngly compromised because of the growing occurrence of ntibiotic resistance among *Shigella* spp. In addition, the ost of treating shigellosis with antibiotics, particularly in he developing world, is impractical and stresses the need or an efficient vaccine against this disease. Currently, owever, there is no vaccine available that can provide dequate protection against the many different serotypes f *Shigella*. Therefore, both the development of new treat- ents and the design of innovative vaccines for the revention of shigellosis rely on an improved under- tanding of the pathogenesis of the disease. Our knowl- dge of the pathogenesis of *Shigella* infection thus far and hat we hope to learn in the future has and continues to epend on our ability to model the infection *in vitro* and to validate these models with *in vivo* studies. This review outlines our current understanding of the pathogenesis of *Shigella* infection. Specifically, findings from *in vitro* systems will be compared to those gained from animal models of shigellosis, while keeping in mind essential features of the disease in humans. This is followed by a discussion of the possibilities for future research and where we believe further studies are required.

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2. *SHIGELLA* INFECTION—OVERVIEW

Shigella flexneri is a Gram-negative facultatively intracellular pathogen that invades the colonic and rectal mucosae of humans, causing bacillary dysentery. Shigellosis is highly infectious, with ingestion of as few as 100 organisms resulting in disease (Dupont *et al.* 1989), and is transmitted by person-to-person contact or indirectly through contaminated food or water. Shigellosis produces a spectrum of clinical outcomes ranging from watery diarrhoea to classic dysentery characterized by fever, violent intestinal cramps and discharge of mucopurulent and bloody stools. Inflammation of the infected tissue is a key feature of shigellosis. Histopathological studies of colonic biopsies from infected patients reveal inflammatory cell infiltration into the epithelial layer, tissue oedema and eroded regions of the colonic epithelium (Mathan & Mathan 1991).

Since this organism is unable to invade epithelial cells through the apical route, *Shigella* exploits M cells, the specialized epithelial cells in the follicular associated

pithelium (FAE) that overlie lymphoid tissue, to gain ntry into the colonic epithelium (Wassef *et al.* 1989). *M* cells allow intact *Shigella* to traverse into the under- ying subepithelial pocket where macrophages reside. Macrophages engulf *Shigella*, but instead of successfully estroying the bacteria in the phagosome, the macro- hage succumbs to apoptotic death (Zychlinsky *et al.* 992). Prior to cell death, infected macrophages release L-1β through the direct activation of caspase-1 by *higella* (Zychlinsky *et al.* 1994). The pro-inflammatory ature of this cytokine results in the recruitment of poly- norphonuclear cells (PMNs) that infiltrate the infected te and destabilize the epithelium (Perdomo *et al.* 994*a*,*b*). Loss of integrity of the epithelial barrier allows nore bacteria to traverse into subepithelial space and ives these organisms access to the basolateral pole of the pithelial cells (Mounier *et al.* 1992). *Shigella* can then nvade the epithelial cells lining the colon, spread from ell to cell and disseminate throughout the tissue. Cyto- ines released by infected epithelial cells attract increased umbers of immune cells to the infected site, thus ompounding and exacerbating the inflammation.

3. INVASION OF EPITHELIAL CELLS

The link between epithelial cell invasion and expres- ion of the virulent phenotype of *Shigella* was first made in 964 (LaBrec *et al.* 1964). The Serény test, which is the ldest animal model of shigellosis, was used as a model to est *Shigella* invasiveness (Serény 1955). This assay consists f inoculating a suspension of bacteria into the kerato- onjunctival sac of guinea-pigs or mice. Pathogenic *higella* invade the conjunctival epithelium causing onjunctivitis and keratitis. This model proved useful for dentifying avirulent mutants of *Shigella* that are incapable f expressing the invasive phenotype. However, the lack f specificity of the response makes it impossible to discri- minate among the various phenotypes of *Shigella* ncluding invasion of epithelial cells, cell-to-cell spread nd the initiation of an inflammatory response.

Cultured epithelial cell lines have greatly aided the study f the host-cell events involved in cell invasion by *Shigella*. Examination of *Shigella*-infected cells by microscopic nethods has defined the entry event as a macropinocytic- ke process that results in massive induction of host cell nembrane ruffling—changes which are reminiscent of hose elicited by growth factors. In the case of invading *higella*, however, the membrane ruffles are confined to the te of bacterium–cell interaction. Cytoskeleton-mediated nembrane extensions are observed to rise up from the rface of the cell and these projections eventually fuse to ngulf the bacterial body (figure 1).

Studies of epithelial-cell–*Shigella* interactions often use oorly differentiated and non-polarized epithelial cell nes, such as HeLa or HEp-2 cells, grown in tissue culture asks. However, more sophisticated systems using human ntestinal cell lines grown on permeable filter supports ith distinct upper (lumenal) and lower (basal) chambers ave been employed. Growing intestinal Caco-2 or T84 ell lines in this way allows the cells to grow as columnar pithelial cells with a more or less organized brush border depending on the cell line) and to polarize with distinct pical and basolateral membranes separated by inter-

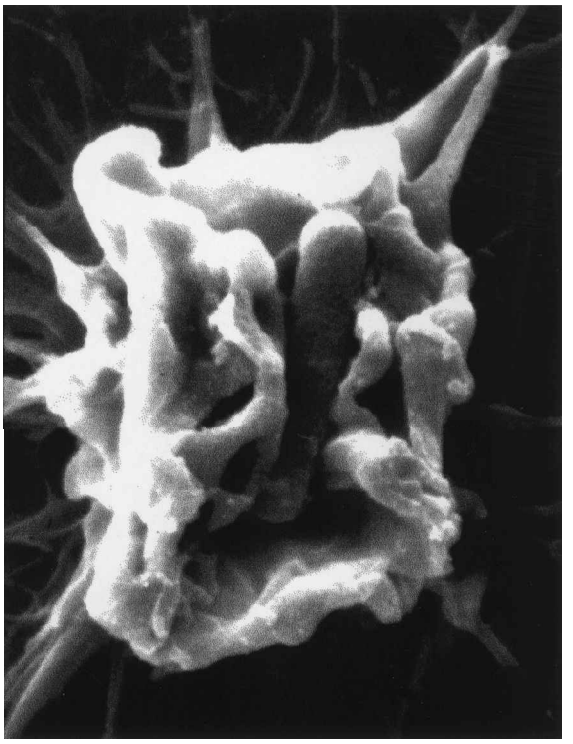


Figure 1. Scanning electron micrograph of *Shigella flexneri* inducing membrane ruffles on the surface of an epithelial cell prior to its uptake. Photograph is courtesy of Dr Ariel Blocker (Institut Pasteur, France) and Dr Roger Webf (European Molecular Biology Laboratory, Heidelberg, Germany).

cellular tight junctions. Bacterial infection of the apical surface of cultured intestinal cells grown in this way more closely mimics infection of the human intestinal epithelium. Using this system, a surprising observation was noted as apically infecting *Shigella* cannot invade polarized cells. Only when intercellular junctions are disrupted by treat- ment of the cells with ethylene glycol-bis (beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) are *Shigella* able to invade the filter-grown Caco-2 cells. These studies indi- cated that *Shigella* enter polarized Caco-2 cells almost exclusively from the basolateral pole (Mounier *et al.* 1992).

A methodological step forward was made in the study of molecular mechanisms of bacterial invasion when it was realized that the aminoglycoside antibiotic genta- micin is membrane impermeable and thus bacteria that are able to enter host cells survive antibiotic treatment of an infected monolayer. This lack of accessibility of gentamicin to intracellular bacteria forms the basis of the 'gentamicin protection assay' whereby the capacity of an organism to invade eukaryotic cells can be assessed repro- ducibly and quantitatively. Using this assay, a number of genes necessary for *Shigella* entry have been identified by analysing mutants defective in surviving gentamicin treat- ment. Genes encoding bacterial factors required for *Shigella* entry reside on a 200 kb virulence plasmid of wild-type *S. flexneri*. Strains lacking the plasmid are non- invasive *in vitro* and also avirulent in animal models of shigellosis. The effectors of *Shigella* entry are the so-called 'invasion plasmid antigens' or Ipa proteins which are encoded in a 30 kb 'entry region'. This region is composed of two adjacent loci transcribed in opposite directions. One locus is essentially composed of the *ipa* operon,

which encodes four secreted proteins, IpaB, IpaC, IpaD and IpaA, which are the effectors of bacterial entry *in vitro*. Mutations in the genes encoding IpaB, IpaC and IpaD proteins render the bacteria non-invasive in cell culture systems and are also avirulent in animal models; these *Shigella* mutants are unable to provoke keratoconjunctivitis in guinea pigs (Ménard *et al.* 1993). An IpaA mutant of *Shigella* maintains a 10% invasion efficiency as assessed *in vitro*; however, it is unable to induce fluid accumulation in rabbit ligated loops, suggesting that the full complement of Ipa proteins are necessary for efficient translocation of *Shigella* across the epithelial barrier and the initiation of an inflammatory response. The other locus in the entry region, the *mxi*/*spa* locus, comprises genes that encode for a type-III secretion apparatus, an evolutionary conserved bacterial system that is responsible for the host-cell contact-dependent secretion of the Ipa proteins, presumably into the host cell's cytoplasm (for a review, see Hueck 1998). Mutations in the genes encoding the type-III secretion system are also avirulent based on the Serény test, due to their inability to invade (Sasakawa *et al.* 1988).

The detailed mechanisms by which the Ipa effector proteins bring about *Shigella* invasion have not yet been fully defined. The Ipa proteins are synthesized and stored within the bacterial body and are secreted through the type-III secretion system upon contact with the host cell (Ménard *et al.* 1994). A complex formed by the association of IpaB and IpaD is thought to regulate the flux of Ipa proteins through the secretion system (Ménard *et al.* 1996). Once secreted, IpaB and IpaC form a complex interacting with the epithelial cell membrane. This complex forms a pore through which it is presumed the other Ipa proteins are translocated into the host cytoplasm (Blocker *et al.* 1999). IpaC and IpaA appear to orchestrate the cytoskeletal rearrangements necessary to direct uptake of the organism into the normally non-phagocytic epithelial cell (Tran Van Nhieu *et al.* 1997, 1999; Bourdet-Sicard *et al.* 1999). Once the *Shigella*-containing vacuole is formed within the infected cell, IpaB mediates lysis of the vacuole and the bacterium is then free in the cytosol (High *et al.* 1992).

4. CELL ADHESION RECEPTORS AND *SHIGELLA* ENTRY

A number of cell adhesion receptors have been implicated in *Shigella* entry into epithelial cells. A secreted complex of IpaB–C–D has been shown to bind α5β1 integrins *in vitro* and this interaction appears to play a role in *Shigella* entry since overexpression of α5β1 in Chinese hamster ovary cells leads to efficient invasion compared to non-transfected cells (Watari *et al.* 1996). α5β1 integrins are present on the basolateral surface of epithelial cells where they mediate interaction with the extracellular matrix. Thus, the location of integrins is in agreement with studies indicating that the basolateral membrane is the point of entry of *Shigella* into epithelial cells. Since β1 integrins interact with the actin cytoskeleton through the carboxy-terminal moiety of the β1 subunit, it was suggested that the binding of *Shigella* to integrins induces cytoskeletal rearrangements leading to the formation of focal adhesion-like structures. Consistent with this idea, the small GTPase Rho, which is important in stress fibre and focal adhesion formation, was shown to be necessary for invasion of epithelial cells by *Shigella* (Adam *et al.* 1996; Watari *et al.* 1997). Additionally, a number of proteins normally associated with focal adhesions are recruited to the site of *Shigella* entry. The focal adhesion components vinculin and ezrin have been shown to be associated with the *Shigella*-induced entry structure (Tran Van Nhieu *et al.* 1997; Skoudy *et al.* 1999*b*).

More recently, another cell adhesion receptor was shown to play a role in *Shigella* entry into epithelial cells. The IpaB–C complex binds to CD44 during *Shigella* entry of HeLa epithelial cells and this interaction also appears to be important for invasion since blocking antibodies to CD44 significantly reduce the uptake of *Shigella* into cells (Skoudy *et al.* 1999*a*). CD44 is the receptor for hyaluronan, a component of the extracellular matrix. Thus, CD44, like β1 integrins, is likely to be expressed on the basolateral membrane of epithelial cells, putting it in an optimal position for the putative interaction with translocated *Shigella*. Through its cytoplasmic domain, CD44 interacts with ezrin, a protein belonging to the ezrin–radixin–moesin (ERM) family of proteins that act to crosslink the plasma membrane and the actin cytoskeleton. ERM proteins are thought to be important in the dynamic regulation of cell shape as they accumulate underneath the plasma membrane in subcellular structures such as microvilli, cell–cell contact sites as well as membrane ruffles, filopodia, microspikes and lamellipodia. Ezrin is also enriched in the cellular protrusions that engulf invading *Shigella* (Skoudy *et al.* 1999*b*). Moreover, it was shown that the dynamic regulation of the cytoskeleton potentially through ezrin is important for *Shigella* entry. Transfection of cells with a dominant negative form of ezrin significantly reduced the ability of *Shigella* to invade. A role for Rho GTPases is again indicated here since Rho can regulate the association of ERM proteins with the plasma membrane (Takahashi *et al.* 1997).

Unfortunately, *in vivo* validation of the above-mentioned *in vitro* experiments is lacking. Therefore, the role played by either of α5β1 integrins or CD44 in *Shigella* invasion *in vivo* is unknown and difficult to test directly. However, it is likely that *Shigella* entry into host epithelial cells is the result of a coordinate action of many different signal transduction pathways and the use of any particular receptor may be redundant. In the case of integrins, only the Ipa complex itself and not the bacterium bind to integrins, questioning the role of this interaction in *Shigella* entry. Additionally, cells that are deficient in either integrins or CD44 are only partially defective in their ability to be invaded by *Shigella* (Skoudy *et al.* 1999*a*). It has been speculated that the IpaB–C complex transiently associates with either integrins or CD44 and this increases the efficiency by which these proteins are inserted into the host membrane where they then act as a pore through which the effector Ipa proteins travel into the host cytoplasm. Clearly, however, these proteins can be inserted into host membranes and *Shigella* can invade cells even in the absence of these cell-adhesion receptors. This brings about the question of whether or not adhesion is a necessary prerequisite to epithelial cell invasion by *Shigella*. So far, adherence of *Shigella* to epithelial cells has not been fully described and the recent sequencing of the

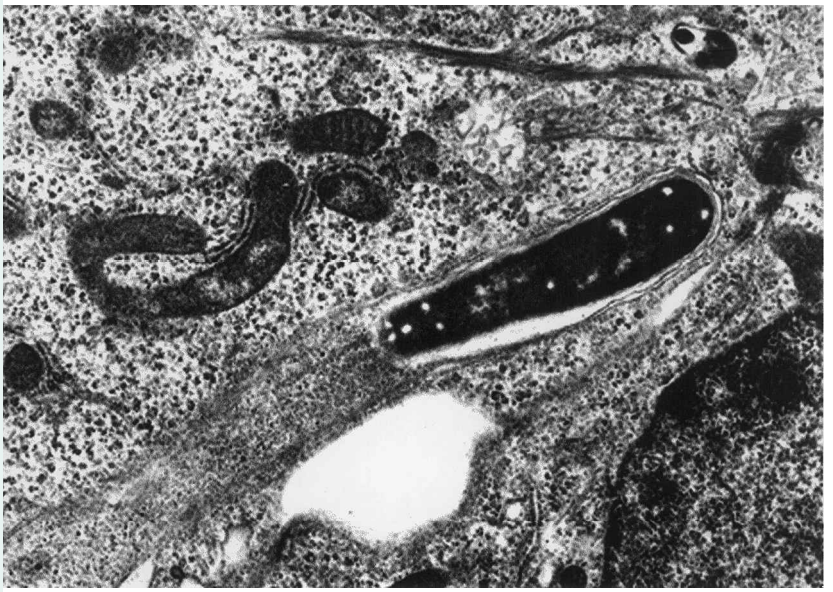


Figure 2. Transmission electron micrograph of a *Shigella flexneri*-mediated protrusion being taken up by a neighbouring cell. Note the dense accumulation of actin behind the moving bacterium. Photograph is courtesy of Dr Michelle Rathman (Institut Pasteur, France).

irulence plasmid has not identified any putative adhesins n *Shigella* (C. Parsot, personal communication). More-ver, the ability of *Shigella* to enter cells of many different pecies argues against any particular species-specific eceptor necessary for invasion. Secretion and insertion of he IpaB–C pore into host membranes may be the rate-miting step in *Shigella* invasion and a receptor as such is otentially unnecessary. This, however, is speculation nce an exhaustive search for a putative *Shigella* adhesin waits further research.

5. INTRA- AND INTERCELLULAR DISSEMINATION

Once inside the host cell cytoplasm, *Shigella* lyse the membrane-bound vacuole and escape into the cytoplasm. A direct consequence of this contact with the intracellular milieu is intracellular motility. The outer membrane protein, IcsA, is necessary and sufficient to direct actin-based motility of *Shigella* within the host cytoplasm (Bernardini *et al.* 1989). The functional role of IcsA in actin-based motility and the cellular partners involved have been recently reviewed (Sansonetti *et al.* 1999*a*). Intracellular *Shigella* use cytoskeletal components to propel themselves inside the infected cell and when contact occurs between the moving organism and the host cell membrane, cellular protrusions are formed. These protrusions are then engulfed by the neighbouring cell thus permitting cell-to-cell spread of *Shigella* without the bacterium ever leaving the confines of the host epithelial layer (figure 2).

Assays to study cell-to-cell spread have centred on two techniques, the plaque assay and the infectious foci assay. In the plaque assay, epithelial cells are infected with wild-type *Shigella* and following a period of incubation, medium containing gentamicin and agarose is added to the infected cell monolayer in order to restrict reinfection of cells from bacteria in the culture media. In this way, bacteria must spread through the epithelial layer by passing from one cell to the next. Two to three days later, the agarose plug is removed and plaques can be observed in the epithelial monolayer. These plaques correspond to points of initial cellular infection and the resulting destruction and clearing of infected cells (Oaks *et al.* 1985). Using this assay, a number of mutants have been identified that are deficient in their ability to spread from cell to cell and of these, IcsA has been best characterized. The ability of IcsA to induce actin polymerization is equally required for intracellular and intercellular spread. Moreover, the IcsA phenotype is extremely relevant during infection *in vivo*. Monkeys infected with an IcsA mutant of *Shigella* develop only mild dysenteric symptoms and show limited histopathological lesions of the colonic and rectal mucosae (Sansonetti *et al.* 1991). These findings stress the requirement for intercellular spread for full virulence of *Shigella* during infection *in vivo* and perhaps point to the role of the epithelial cell in the development of widespread inflammation (discussed in § 9).

E-cadherin, a key protein involved in intercellular adhesion, has been shown to be an important cellular component involved in the intercellular spread of *Shigella*. Cell–cell contacts were thought to be necessary for intercellular spread because of the observation that *Shigella* passed from one cell to the next essentially at sites of the intermediate junctions in Caco-2 cells (Vasselon *et al.* 1992). In addition, transmission electron microscopic observations of various epithelial cell lines showed that passage of *Shigella* protrusions from one cell to the next occurred at sites where the two cells were closely apposed, suggesting that cell–cell contacts were involved. To test this directly, the infectious foci assay was developed. In this assay, cells are infected with *Shigella* for a period of time and subsequently trypsinized and seeded at a very low density with a population of uninfected cells that are either cadherin-negative or stably expressing cadherin. In the cells expressing cadherins, *Shigella* is efficiently transmitted from the originally infected cells to the neighbouring cells such that large areas of the monolayer are observed to be infected. In contrast, cells that are deficient in cadherins do not transmit *Shigella* and the infection remains limited to the index cells only. Therefore cell–cell contacts, dependent on the expression of cadherins, are necessary for intercellular spread of *Shigella* (Sansonetti *et al.* 1994). Further research is required to identify whether or not *Shigella* interacts

irectly with proteins at this junction to bring about rotrusion formation. Additionally, the role played by ntermediate junctions in the pathogenesis of *Shigella* uring infection *in vivo* needs to be addressed. Again, ecause of the complexity of the system, such *in vivo* vidence is difficult to obtain and will rely on the develop- nent of novel model systems. In the meantime, however, omplementary techniques, such as the expression of ominant negative proteins, the use of specific inhibitors s well as cell lines deficient in certain proteins, certainly nds credence to these *in vitro* findings.

6. M CELLS: PORTS OF ENTRY INTO THE HOST EPITHELIUM

One of the key events in the pathogenesis of enteroinva- ve bacterial infections is the penetration of the intestinal pithelium. Since *Shigella* cannot enter epithelial cells via ne apical pole, it uses M cells to gain entry into the host pithelium. In fact, many Gram-negative bacteria that ause enteric disease, including *Salmonella* and *Yersinia*, ave been shown to preferentially cross the epithelium via pecialized antigen sampling cells called M cells (for a eview, see Sansonetti & Phalipon 1999). M cells, which tands for membranous or microfold cells, are modified pithelial cells found within the FAE overlying lymphoid ollicles. These follicular lymphoid structures are scattered roughout the small intestine in aggregates known as 'eyer's patches and in the colon and rectum as isolated olitary nodules. M cells are relatively rare, constituting ss than 0.1% of epithelial cells present in the lining of ne intestine and can be identified morphologically due to ne fact that they display (i) a poorly differentiated brush order compared with neighbouring absorptive epithelial ells, and (ii) an irregular basolateral membrane border ontaining invaginated lymphocytes. M cells have a igh endocytic activity which serves to transport soluble nd particulate lumenal antigens across the cytoplasm nd deliver them intact to the antigen-processing and resenting cells in the underlying follicle (Neutra *et al.*). t is perhaps surprising that a cell so rare in the intestinal pithelium can be the target of entry for many different athogens. How then do these pathogens seek out M cells nd use these cells to enter the host epithelium? It has een suggested that the lack of both mucus and a well- eveloped glycocalyx over the FAE facilitate non-specific nteractions of pathogenic organisms with M cells. ncreased hydrophobic interactions may be favoured and his could be the primary step that precedes what is likely o be a non-specific transport mechanism. In fact, it has een shown that lectins, positively charged particles and ydrophobic beads, all of which bind to the membrane urface of M cells, are transported with increased effi- iency (reviewed in Jepson *et al.* 1996). M cells may also xpress characteristic surface molecules that could serve s specific receptors for pathogens. For example, M cells xpress characteristic glycoconjugates, which vary epending on the species and the location in the intestine Giannasca *et al.* 1994; Lelouard *et al.* 1999). Although no pecific receptor engaged by a bacterial adhesin or nvasin has been identified, such a receptor may account or tissue tropism of a particular pathogen as well as its fficient uptake into the FAE.

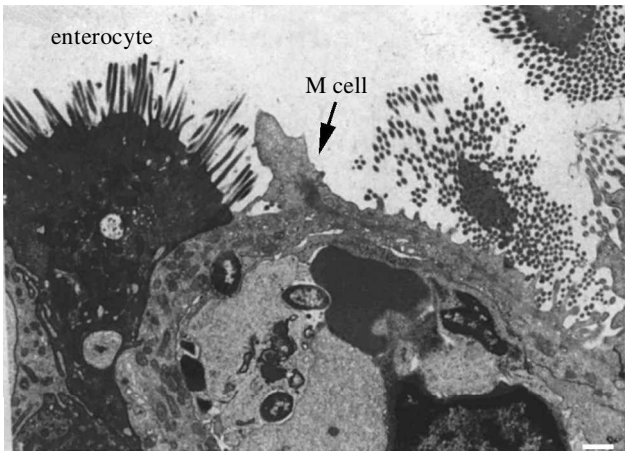


Figure 3. Transmission electron micrograph of *Shigella flexneri* crossing the intestinal epithelium by an M cell. Photograph adapted from Sansonetti & Phalipon (1999).

7. M CELLS AND ENTRY OF *S. FLEXNERI* INTO THE HOST: *IN VIVO* EVIDENCE

The first indication that *S. flexneri* exploits M cells to enter the host epithelial layer came from studies using a rabbit ligated ileal loop model. In this model, animals are anaesthetized and the intestine is externalized at laparotomy. Sections of ileum are carefully ligated to preserve the existing vasculature and, subsequently, a large inoculum of bacteria, usually 10^9 colony forming units (CFU) ml^{-1}, is injected into the intestinal loop. Invasive and inflammatory properties of the organisms can be observed following sacrifice of the animals at a given time post-infection (usually 2–8 h). Histological studies and measurements of fluid accumulation within the infected loop can be conducted. By isolating ileal loops with grossly identifiable Peyer's patches, the role of the FAE in the initial steps of epithelial translocation by *Shigella* has been assessed. Wild-type *S. flexneri* was readily detected in the dome epithelium of the FAE, whereas very few organisms were observed within the villus epithelium. In addition, when the infected loops were incubated for longer time periods, ulcerations were observed preferentially over the dome regions of Peyer's patches suggesting that the FAE was the primary site of entry (Wassef *et al.* 1989). These findings were reconfirmed using the rabbit ileal loop model in a study indicating that threefold more bacteria were present within the infected tissue if Peyer's patches were present within the loop compared with those loops that lacked lymphoid follicles (Perdomo *et al.* 1994*b*). Figure 3 shows wild-type *Shigella* crossing the epithelial barrier by an M cell.

These findings were also confirmed in the macaque monkey model of shigellosis. Macaques in particular are one of the few animals that develop a dysentery-like disease following oral or gastric inoculation of *Shigella*, although a dose of 10^{10} organisms is typically required for the development of disease. Using this model, it was observed that when monkeys were infected with an *icsA* mutant of *S. flexneri*, which does not spread intra- or intercellularly, animals do not develop clinical symptoms but small ulcers corresponding to the presence of lymphoid follicles are observed on the colonic lining (Sansonetti *et al.* 1991). These findings suggest that the *icsA* mutant is

apable of entry into the FAE but owing to its inability to pread from the initial entry site, only a local ulceration t the point of the FAE is observed. These findings onfirm that the FAE serves as the site of bacterial entry nto the epithelium and also reiterates the role of intra- nd intercellular spread in the development of widespread nflammation during wild-type *Shigella* infection.

Another important observation made using the ileal oop model was that a significantly greater number of ild-type *Shigella* were found in the dome epithelium ompared with non-pathogenic strains or heat-killed rganisms, suggesting that the presence of virulence actors play a role in the increased uptake of the wild- ype strain into the FAE (Wassef *et al*. 1989). This bservation was further characterized using strains of . *flexneri* expressing either an invasive or an adhesive but on-invasive phenotype in the rabbit ileal loop model. he adhesiveness of the latter strain is mediated by the xpression of an *Escherichia coli* adhesin that mediates ttachment of the organism to rabbit M cells (Inman & antey 1984). By immunostaining for lipopolysaccharide LPS), it was shown that the amount of bacterial material ssociated with the FAE and the dome of the lymphoid ollicle was essentially equivalent in loops infected with ither the adhesive–non-invasive or the invasive strain, hereas very few control organisms, i.e. non-adhesive nd non-invasive, could be isolated from similarly nfected loops (Sansonetti *et al*. 1996). These data suggest hat either specific adhesion to M cells or an invasive apacity to enter M cells is required for an organism to e transported through the epithelium and into the ubepithelial space. What was also clear from this study as that once the bacteria gain access to the subepithelial pace they have very different fates depending on their irulence capacities. Whereas infection with wild-type, lly invasive *Shigella* results in rapid inflammation and ubsequent destruction of the FAE, the adhesive yet non- nvasive strain is sequestered and destroyed within ysosomes of macrophages present within the dome.

Although the animal models discussed above have been seful for studying some aspects of *Shigella*–M-cell inter- ctions, there are significant drawbacks and their direct elevance to human infection can be questioned. Most bvious is the fact that oral or gastric inoculation of abbits does not lead to dysenteric symptoms. Also, shigel- osis in humans is a disease of the distal colon and ectum, whereas in the rabbit model it is the ileum that is udied. Therefore, this model does not take into account he tissue specificity that is seen in human infection thus gnoring the potential of a specific interaction between *higella* and M cells of the human colon. In addition to aving ethical and financial drawbacks, the macaque odel is also not ideal since the infectious dose required or the animals to develop dysentery is ten million to 100 illion times higher that the infectious dose in humans. he questionable relevance of such a model is particularly pparent in the context of testing the tolerance of attenu- ted vaccine candidates or doing challenge experiments n vaccinated animals.

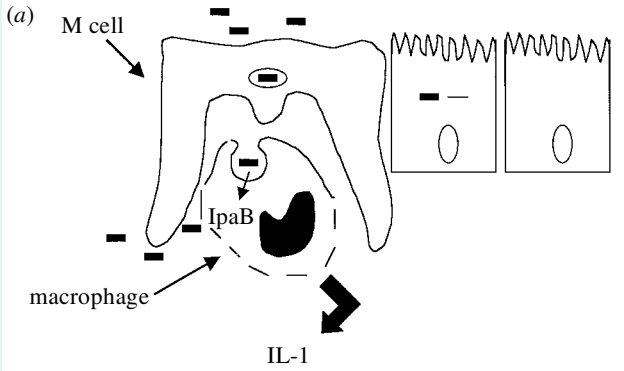
Despite these drawbacks, however, many of the obser- ations made in animal studies do correlate with what we now from human infections with *Shigella*. In fact, clinical bservations of patients suffering from shigellosis support the idea that the FAE is the primary route of entry of *Shigella* into the host tissues. In patients examined endoscopically within two days of the start of infection, early inflammatory lesions resembling aphtoid ulcers are present in the rectum and distal colon, and on histopathological observation these lesions are found to correspond to lymphoid follicles (Mathan & Mathan 1991). Additionally, inflammation is observed to be confined to follicular regions of the rectum and distal colon early in the course of infection, but is later detected in the surrounding villi and can be seen to extend proximally (Islam *et al*. 1994).

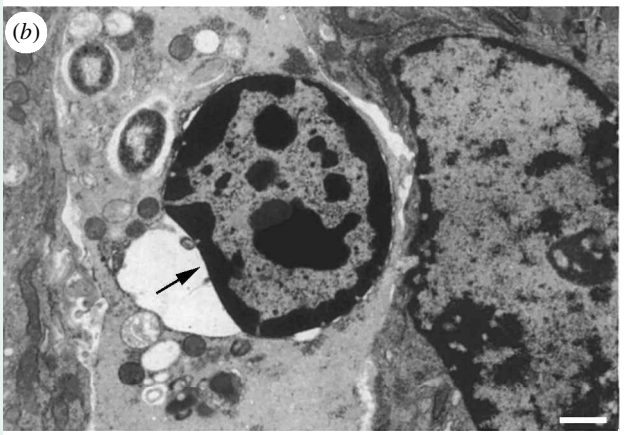
There are a number of aspects of M cell–*Shigella* interactions, however, that cannot be addressed adequately using these *in vivo* systems and therefore require *in vitro* modelling. Recently, such a model was developed in which Caco-2 cells, a human intestinal cell line, were induced to switch to an M-cell phenotype when co-cultured with lymphocytes isolated from the Peyer's patch (Kernéis *et al*. 1997). Using this model, it was shown that *Vibrio cholerae* O:1, a non-invasive pathogen transported exclusively by M cells (Owen *et al*. 1986), could also be transported across the model epithelium by the *in vitro*-induced M cells. This model will assist studies into *Shigella*–M-cell interactions and may help to identify specific receptors or adhesive factors on M cells that facilitate the uptake of *Shigella* by these cells. Additionally, this model will allow the determination of the virulence factors of *Shigella* that are necessary for entry and transport across M cells.

At later time-points of infection, inflammation disrupts the integrity of epithelium and this may be a secondary means by which pathogenic *Shigella* translocate across the epithelial barrier in order to reach the basolateral pole of epithelial cells, which they can then efficiently invade. The possibility of this mode of *Shigella* translocation was modelled *in vitro* (Perdomo *et al*. 1994*a*). This system was again based on the culture of monolayers of human intestinal cells on filter supports; however, another layer of complexity to this system was added so that the early immune responses to invasive bacteria could be investigated. In this system, isolated human PMNs are added to the basolateral side of polarized T84 cells, which are then infected apically with pathogenic *Shigella*. A rapid paracellular transmigration of the PMNs which disrupts the barrier function of the epithelium is observed, as measured by a drop in transepithelial electrical resistance. Subsequent to these events, *Shigella* is able to pass through the disrupted tight junctions and thus gains access to the basolateral pole of the cells (Perdomo *et al*. 1994*a*). These studies suggest that lumenal *Shigella* can induce epithelial cells to produce potent chemotactic signals that elicit transepithelial transmigration of PMNs. In fact, the epithelial cell has been shown to play a significant role in innate immunity against enteroinvasive bacterial infections (Jung *et al*. 1995) and, in the case of *Shigella* infection, is important in intiating the inflammatory response (Sansonetti *et al*. 1999*b*; see § 9).

8. MACROPHAGE APOPTOSIS IN RESPONSE TO *SHIGELLA* INVASION

Bacteria that have crossed the epithelial layer via M cells are likely to be phagocytosed by resident macrophages





igure 4. (*a*) Model of *Shigella flexneri* penetration of the ıtestinal epithelium by M cells and subsequent contact with ıe underlying macrophages at the site of the follicular ymphoid tissue. *Shigella* is phagocytosed by resident ıacrophages; however, the organism escapes from the phago- ytic vacuole and induces macrophage apoptosis via ıteraction of bacterial IpaB with host cell caspase-1. ctivated caspase-1 cleaves and activates pro-IL-1 that is eleased in large quantities from the dying macrophage. *b*) Apoptosis of a *Shigella*-infected macrophage *in vivo*. Arrow oints to the condensation of chromatin at the periphery of ıe nucleus which is a characteristic of apoptotic cell death. hotograph adapted from Sansonetti & Phalipon (1999).

ithin the subepithelial dome overlying the lymphoid folli- les (see figure 4*a*). The uptake of *Shigella* by macrophages *in itro* does not require the virulence plasmid and presumably ccurs by normal phagocytic mechanisms. The fate of *. flexneri* following phagocytosis was first studied in the late 980s using the murine macrophage J774 cell line (Clerc *et l.* 1987). It was noted that uptake of *Shigella* resulted in lysis f the phagocytic vacuole and rapid killing of the infected ell. It was not until five years later, however, that macro- hage cell death was shown to occur by apoptosis Zychlinsky *et al.* 1992; figure 4*b*). Apoptosis was not seen ith plasmid-cured strains, which led to the identification f the plasmid-encoded protein, IpaB, as the mediator of ell death (Zychlinsky *et al.* 1994). IpaB gains access to the ytosol, where it binds and activates caspase-1, also known s interleukin (IL)-1-converting enzyme (Chen *et al.* 1996). ctivation of caspase-1 is absolutely required for *Shigella*- nduced apoptosis, since cell death is not seen in caspase-1 nockout mice (Hilbi *et al.* 1998). The downstream events romoting apoptosis following caspase-1 activation by IpaB re unknown.

Apoptosis is generally considered to be an immunologically silent cell death process unaccompanied by inflammation, however, this is not the case with caspase-1-dependent apoptosis. Caspase-1 cleaves and activates the pro-inflammatory cytokines IL-1β and IL-18 (Ghayur *et al.* 1997). Murine macrophages have been shown to release large amounts of mature IL-1β during the *Shigella*-induced apoptotic process (Zychlinsky *et al.* 1994). Given that mucosal inflammation is the hallmark of shigellosis, these observations made largely in murine macrophage cell lines prompted the search for evidence that apoptosis and the consequent cytokine production play a role in *Shigella* infection *in vivo*.

Apoptotic cells have been identified in the subepithelial dome and lymphoid follicles in a rabbit ligated ileal loop model of *Shigella* infection (Zychlinsky *et al.* 1996). Apoptotic cells were not seen in the mucosa when challenged with plasmid-cured *Shigella* or plasmid-cured strains transfected with an *E. coli* adhesin, which allowed the bacteria to penetrate into the subepithelial space in comparable numbers to the wild-type *Shigella*. Apoptotic cells have also been seen in rectal mucosal biopsies from patients acutely infected with *Shigella* (Islam *et al.* 1997). Together these observations provide evidence for apoptosis *in vivo* during *Shigella* infection, and suggest that this phenomenon is due to the presence of the virulence plasmid.

There have recently been some reports that *Shigella* can kill macrophages by an alternative mechanism termed oncosis, and that this process does not involve caspase-1 (Fernandez-Prada *et al.* 1997; Nonaka *et al.* 1999). In the latter report a differentiated human monocyte-like cell line, U937, was used to show that *Shigella* infection could result in apoptosis or oncosis depending on the differentiation stimulus used. Evidence of oncosis *in vivo* in *Shigella* infection and whether it contributes towards the disease manifestations has yet to be investigated.

9. ENCOUNTERS WITH THE INNATE IMMUNE RESPONSE

The innate immune response provides an early defence against bacterial infection, which serves to limit bacterial proliferation, localize the infection and also both activate and regulate the subsequent adaptive immune response. Many cell types and soluble proteins, including phagocytic cells (neutrophils, macrophages and dendritic cells), lymphocytes (natural killer (NK) cells and γδ T cells), cytokines (most notably, IL-1, Il-6, IL-12, tumour necrosis factor α (TNFα) and IFNγ) and liver-derived serum proteins such as complement factors contribute towards innate immunity. In addition to these classical immune components, non-immune cells such as epithelial cells recognize and respond to bacterial invasion by producing chemokines that can attract and activate immune cells (Jung *et al.* 1995). The net result of the complex interaction between these many factors is usually manifested as acute inflammation.

The study of the innate immune response in shigellosis has largely focused on the mechanisms involved in regulating the influx of neutrophil into the infected site. The neutrophil response can be separated into two stages: an initial influx focused in the region of the lymphoid follicles; and a later phase of massive neutrophil influx into intestinal

illi and crypts, which produces large areas of epithelial estruction and mucosal ulceration that extend far beyond he initial site of bacterial entry (Mathan & Mathan 1991). t is likely that these two stages reflect different processes. L-1 released from *Shigella*-infected apoptotic macrophages ay be responsible for the first stage; in the rabbit ileal loop nfection model, treatment with an IL-1 receptor antagoist prior to infection with virulent *S. flexneri* significantly ecreased the inflammation and tissue destruction within he lymphoid follicles (Sansonetti *et al.* 1995).

Observations that the second stage of inflammation ccurs at some distance from the follicles and that infected pithelial cells secrete pro-inflammatory cytokines rompted an investigation into the role of IL-8 as a ediator of inflammation in this second phase (Sansonetti *al.* 1999*b*; see figure 5*a* for model). Again using the *in vivo* abbit ileal loop model, a neutralizing anti-IL-8 monolonal antibody was found to considerably reduce the eutrophil influx entering via the lamina propria into the ntestinal villi and to attenuate the consequent epithelial estruction. *In vitro* studies have shown that IL-8 producion by epithelial cells induces neutrophil migration across olarized epithelial monolayers and this can occur with or ithout invasion of the epithelial cells by *Shigella* (Beatty Sansonetti 1997; McCormick *et al.* 1998). Thus, bacterial nteraction with epithelial cells appears to be a requirement for this second phase. The rapid extension of inflamation to sites distant from the follicles stresses the mportance of cell-to-cell spread by *Shigella*, and is further upported by experimental *Shigella* infection of macaque onkeys with the *icsA* mutant, capable of epithelial cell nvasion but unable to spread from one cell to another Sansonetti *et al.* 1991). The inability of the *icsA* mutant to pread through the epithelial layer restricts the contribuion of epithelial chemokine release and consequently mits the inflammation seen in infected animals.

It has been noted that blocking the neutrophil influx sing anti-β1-integrin antibodies, IL-1 receptor antagoists or anti-IL-8 antibodies, all result in decreased epihelial destruction implicating the neutrophil rather than *higella* as the direct cause of mucosal damage (Perdomo *et l.* 1994*b*; Sansonetti *et al.* 1995, 1999*b*). Neutrophils can ill opsonized *Shigella in vitro* (Mandic-Muleg *et al.* 1997), nd the neutrophil inflammatory response localizes the acteria to the epithelium. When neutrophil influx is locked, bacteria migrate deep into the lamina propria nd mesenteric blood vessels, confirming the importance f neutrophils in localizing bacterial infection (Sansonetti *al.* 1999*b*). Thus, neutrophil influx appears to be responble for the majority of tissue destruction associated with higellosis, and yet is vital for preventing the systemic pread of bacteria (figure 5*b*).

The murine lung model of shigellosis, although not relevant with regard to the organ specificity of the disease, has een useful for exploring details of the immune and nflammatory components, as well as some aspects of the ystemic and local immune response against *Shigella* infection (Mallett *et al.* 1993; Verg *et al.* 1995). In this model, an noculum of wild-type *Shigella* is administered intranasally esulting in invasion of the tracheo-bronchial tract esulting in an inflammatory broncho-tracheo-alveolitis Voino-Yasenetsky & Voino-Yasenetskaya 1961). Using this nodel, the role of cytokines in the innate immune response has been investigated. TNFα and IFNγ are both produced locally during the first 24 hours of infection. Sublethal inoculation into IFNγ knockout mice results in overwhelming local proliferation of bacteria and death, compared with a steady decline in bacterial numbers in wild-type controls (Way *et al.* 1998). Histology of lungs from knockout mice showed an obliterative neutrophilic bronchiolitis suggesting that neutrophils alone, in the absence of IFNγ are unable to clear the infection. The course of infection in αβ and γδT-cell knockout mice was

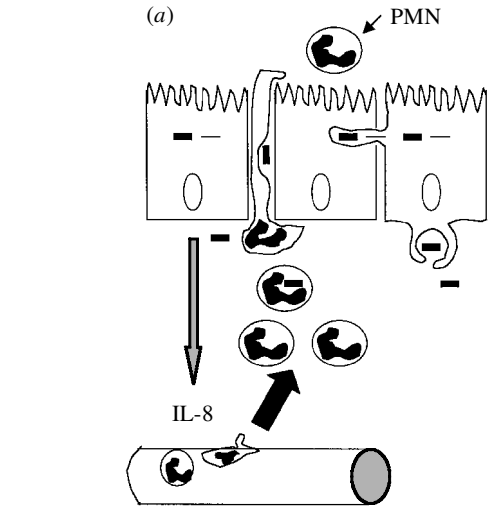


Figure 5. (*a*) *Shigella flexneri*-infected epithelial cells are a source of interleukin-8 (IL-8), a potent chemotactic chemokine that is responsible for the recruitment of PMNs into the infected site. PMNs migrate between adjacent epithelial cells, break intercellular junctions and thus compromise the integrity of the epithelial barrier. This causes destruction of the mucosal surface by allowing invasion of further organisms from the colonic lumen. Conversely, the neutrophil influx is necessary in order to control the proliferation of organisms locally and prevent systemic bacterial dissemination. (*b*) Photographs of intestinal sections stained for LPS from *Shigella flexneri*-infected rabbit ileal loops. The left panel shows a tissue section from an ileal loop infected with *S. flexneri* in a rabbit pre-treated with a control antibody showing abscess formation and local tissue destruction. The right panel shows a tissue section from a rabbit in which IL-8 was neutralized using specific antibodies prior to infection. Although the epithelium is spared, bacterial diffusion into the lamina propria is observed. This stresses the important role for IL-8-mediated neutrophil influx in preventing bacterial translocation. Scale bars, 10 μm. Photographs adapted from Sansonetti *et al.* (1999*b*).

ne same as wild-type controls suggesting neither cell type cted as a source for this cytokine. Conversely, NK-cell-eficient mice had an increased susceptibility implicating ne NK cell as the source of IFNγ. There are probably nultiple roles for IFNγ, including inhibition of bacterial roliferation within epithelial cells (Way *et al.* 1998), nhanced macrophage killing of bacteria and perhaps nhibition of macrophage apoptosis induced by *Shigella* Hilbi *et al.* 1997).

10. THE ADAPTIVE IMMUNE RESPONSE

During the course of infection, *Shigella* exists in both an xtracellular and intracellular location. This implies a equirement for both humoral and cellular immune esponses for effective sterilizing immunity. The fact that nice do not display intestinal infection following challenge vith *Shigella* has hampered study of the adaptive immune esponse. Some information, however, has been obtained rom study of the murine pulmonary inoculation model nd serological studies of infected humans. In the ulmonary model, isotype-specific secretory IgA-anti LPS ntibodies targeted to the mucosa from subcutaneous hybri-omas, provides protection against challenge with a lethal ose of organisms (Phalipon *et al.* 1995). This underscores ne importance of local IgA in providing protection, and upports observations in humans suggesting that protective mmunity is isotype specific and therefore directed pre-ominantly against LPS (DuPont *et al.* 1972). Using the ulmonary challenge model, immunized mice were used to efine the characteristics of a protective humoral response. ublethal infection induces local IgG and IgA responses irected against LPS, and some Ipa proteins, but responses re slow to develop (Verg *et al.* 1995). Short-lived, protective, erotype-specific humoral responses have been generated, lthough this predominantly consists of an IgM response nd is T-cell independent (Way *et al.* 1999*a*,*b*). Again, it is ot clear whether these results accurately reflect the situa-on in the intestinal mucosa.

Our understanding of how the mucosal immune system anages any Gram-negative bacteria, including *Shigella*, to btain LPS in a form that can be presented to B, and erhaps T, cells for induction of a high-affinity IgA response minimal. Recently, it was shown that *Shigella* LPS can be afficked through polarized intestinal cells and thus poten-ally processed and presented in an immunologically ctive form (Beatty *et al.* 1999). Lipoglycans such as LPS are learly dealt with differently from proteins, but apart from bservations that CD1-restricted CD4–CD8 double nega-ve T cells can be generated, reacting with mycobacterial poglycans, there is little information (Porcelli & Modlin 999). There is an urgent need for studies into the immune esponses against bacterial LPS. The situation with cellular mmunity in shigellosis is equally uncertain. T-cell clones ave been produced against *Shigella* (Zwillich *et al.* 1989) nd activated T cells have been isolated from the blood of atients with shigellosis, but their function is unknown Islam *et al.* 1995, 1996).

11. CONCLUSION AND PERSPECTIVES

Our understanding of the pathophysiology of shigel-osis is largely based on studying the invasion of epithelial cell monolayers and macrophages *in vitro*, and the experimental infection of exteriorized rabbit ileal loops. Information has also been obtained from the murine pulmonary model and from rectal biopsies of macaque monkeys and humans after experimental and natural infection, respectively. The fact that *Shigella* does not cause intestinal infection in mice, which denies the use of the many murine-specific reagents and genetic manipulations, has probably inhibited a more detailed investigation of the cytokine and cellular mechanisms involved. Nonetheless, the application of knockout mice in the murine lung model of shigellosis has added, and will continue to add, to our knowledge of the innate and adaptive immune responses to *Shigella* infection. Future studies using transgenic animals expressing human-specific factors will also open up new possibilities for investigating the immune response in shigellosis.

In vitro and *in vivo* studies have allowed the formulation of a detailed model of the disease process, however, many questions still remain to be addressed. An analysis into the timing of the inflammatory response in terms of the cell types and mediators that are recruited and secreted at the site of infection needs to be conducted. For example, it will be important to determine the relative contribution of resident macrophages versus newly recruited monocytes/macrophages to the disease process and which inflammatory mediators are responsible for this induction during infection *in vivo*. Improved techniques that combine multiple immune staining and confocal microscopy of infected tissue sections will help to identify the early players in the development of inflammation following infection with *Shigella*. These techniques could also be used to observe the fate of bacterial virulence factors, such as LPS, in the infected tissue during the course of infection. Techniques to measure cytokines *in situ* with placement of microdialysis probes in the infected site (Bruce *et al.* 1999) have the potential to identify new inflammatory mediators and perhaps point to a novel means of treatment by targeting these molecules and modulating their function during *in vivo* infection.

Another possible research avenue that remains to be explored is the potential for differential gene expression, in both the host and the bacterium, during *Shigella* infection. One example of a host gene specifically upregulated during infection with *Shigella* is IL-8 and its regulation by the eukaryotic transcription factor, NFκB, has recently been demonstrated (Philpott *et al.* 1999). However, a more comprehensive approach to identify the expression of *Shigella*-induced host genes would be the application of DNA microarray technology (reviewed in Khan *et al.* 1999). This approach will lead to the identification of gene products up- or down-regulated during *Shigella* infection. Additionally, this approach could be used to attribute a particular phenotype to *Shigella* mutants that remain uncharacterized. By comparing the pattern of gene expression from wild-type versus mutant infected cells, a particular function could be ascribed for the gene product missing in these mutants. Conversely, genes expressed in the bacterium during infection of the host could also be examined. The potential to apply techniques such as signature-tagged mutagenesis (Hensel *et al.* 1995) to *Shigella* infection also remains unexplored.

It is unwise to assume that any particular, or indeed a combination of, animal models will reveal all the components involved in producing the human disease. Infections usually exhibit a restricted host range, and in the case of many important human infections such as shigellosis, the disease is essentially confined to humans. Therefore, human-specific factors that allow expression of the disease phenotype probably exist. Furthermore, in the past, bacterial infections have infected the majority of the population and caused significant mortality in children, providing the potential for skewing the surviving population towards genetic expression of factors that probably influence the host response to disease. Such factors are likely to act at the level of the innate immune response and may be represented only in humans. Identification of such factors would shed light on natural resistance and, for example, help to explain why in human *Shigella* the challenge studies, a maximum of only 70% of volunteers get the clinical disease (DuPont *et al*. 1969).

For these reasons, in shigellosis, as much as in any other bacterial infection, there is a need to develop experimental models that can more closely mimic human disease, using human cells and tissues. At present, such models remain in the developmental stage. One possibility is the further development of techniques for maintaining the viability of human tissue samples such as intestinal biopsies, which could be used to study the response of resident cells to invasion of *Shigella*. A second possibility is the refinement of techniques for grafting human mucosal tissues into SCID mice (Yan *et al*. 1993) and then repopulating the bone marrow with autologous bone marrow cells. Infection of such xenografts could then be assessed in the context of both the human intestine and immune system yet would be amenable to the manipulations achievable in the mouse. Ultimately, such studies as those described here will help form a basis of knowledge by which improved treatments and novel vaccine candidates for the prevention of shigellosis will be designed.

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Discussion

C. W. Keevil (*Centre for Applied Microbiology and Research, Porton Down, Wiltshire, UK*). With respect to determining the infectious dose or LD_{50} of a gastro-intestinal pathogen, it may be worth considering the recent publication of James & Keevil (1999). This paper showed that verocytotoxigenic *Escherichia coli* 0157 attaches more avidly to enterocytes, with actin filament rearrangement, when grown microaerophilically or anaerobically rather than aerobically. Regrettably, many laboratories do not consider growing facultatively anaerobic pathogens under physiologically relevant conditions, especially low redox potential, prior to their *in vitro* or *in vivo* challenge studies. Could you comment on what anaerobic inoculum experiments have been performed by laboratories when examining the pathogenesis of *Shigella* spp.? One possible interpretation of some of your present data is that *Shigella* spp. express an anaerobic phenotype capable of enhanced attachment to epithelial cells; once they become intracellular, their phenotype will change in response to local nutrients, particularly oxygen concentration, making them fit for subsequent tissue invasion and dissemination to macrophages.

P. J. Sansonetti. I agree with Dr Keevil that not much attention has so far been paid to the effect of anaerobic growth conditions on the invasive capacity of *Shigella*.

With regard to *in vitro* assays of cell invasion, growth conditions have been selected with the aim of optimizing bacterial entry into cells. It turns out that optimal conditions are the middle exponential phase of growth with aeration achieved by shacking. We are far away from anaerobiosis under such circumstances! Still, in those conditions, bacteria need to be centrifuged over the cell surface in order to achieve an intimate interaction, as no significant adherence system has ever been identified. Our preliminary evidence however, based on the sequence and annotation of the *S. flexneri* virulence plasmid, does not show any gene with a homology indicating a candidate for encoding and adhesin. This does not preclude the possibility that a pathogenicity island on the *Shigella* chromosome may encode an adherence system. In any event, the anaerobic growth conditions definitely need to be tested.

The situation seems different with regard to animal models: none of them, except infection in the macaque monkey, really reflects the situation of colonic infection that prevails in humans. In consequence, when macaque monkeys are infected intraperitoneally, the growth conditions do not really matter as the bacteria first need to survive gastric acidity, then transit through the small intestine and finally establish infection in the colon. Under such neutral conditions, bacteria have time to adapt and express any putative specific adhesin. We believe that the best way to identify this putative adhesin will be a combination of genomics and signature-tagged mutagenesis.