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Molecular and Cellular Bases of Invasion of the Intestinal Mucosa by *Shigella flexneri*

Abstract

Shigella flexneri, a gram-negative bacillus, is a member of the family enterobacteriaceae. It causes bacillary dysentery by invading the human colonic mucosa and inducing an inflammatory reaction. Recent data suggest a model of the infectious process. *In vivo*, bacteria enter into the mucosa *via* M-cells, specialized cells present in the epithelial dome covering the lymphoid follicular structures of the intestinal mucosa. Once inside the submucosa, shigellae encounter resident tissue macrophages, which are infected and programmed cell death (i.e. apoptosis) is rapidly induced in these phagocytic cells. During programmed cell death, the inflammatory cytokine Interleukin-1 (IL-1) is released. It triggers an inflammatory reaction characterized by extravasation of polymorphonuclear (PMN) cells. Inflammation is probably potentiated by the production of other cytokines by epithelial, endothelial and PMN cells. PMN cells migrate through the epithelium into the lumen of the colon, thus destabilizing the integrity of the epithelial barrier. The damaged epithelium allows massive entry of bacteria into subepithelial tissues. Further colonization of the epithelium aggravates inflammation which in turn causes extensive tissue destruction. This colonization process encompasses entry into epithelial cells, escape into the host cell cytoplasm and cell to cell spread. Both the *in vitro* and *in vivo* results that support this model are discussed here.

Introduction

Diarrhea is a major public health issue. About five millions children die annually worldwide of diarrheal diseases (Barua, 1981). *Shigellae* are among the important etiologic agents of these diseases (Sanyal, 1981), causing a bloody diarrhea called bacillary dysentery. In developing countries, *Shigella flexneri* is the major etiological agent of the endemic form of the disease. *Shigella dysenteriae* 1 is less common but causes devastating epidemics. Development of vaccines allowing prevention of bacillary dysentery is a priority (Maurelli & Sansonetti, 1988; Lindberg & Pal, 1993).

The major characteristic of the disease is bacterial invasion of the rectal and colonic mucosa. Histopathological analysis of the recto-colon of patients with shigellosis shows severe destruction of the epithelium characterized by mucosal erosion and inflammation dominated by an infiltration and a major exudation of polymorphonuclear leukocytes (PMN) (Anand *et al.*, 1986; LaBrec *et al.*, 1964; Mathan & Mathan, 1991).

A major property of *S. flexneri* is its capacity to invade eukaryotic cells (LaBrec *et al.*, 1964). After phagocytosis, *Shigella* lyses the phagocytic vacuole and escapes into the cytoplasm (Sansonetti *et al.*, 1986; Maurelli & Sansonetti, 1988). In shigellosis, although very few bacteria can cause dysentery, this microorganism is non-pathogenic in experimental animals (Lindberg & Pal, 1993; Maurelli & Sansonetti, 1988) except macaque monkeys. Paradoxically, *in vitro*, *S. flexneri* can efficiently invade all vertebrate cells tested so far. *Shigella* pathogenicity can be analyzed using cell culture of both explanted cells and established cell lines, as well as *in vivo* models like the rabbit ligated loop assay and the Serény test (Serény, 1955; LaBrec *et al.*, 1964). Rabbits are not naturally sensitive to *Shigella*, but injection of the bacterial pathogen into the lumen of ligated intestinal loops mimics the physiopathology observed in experimental human and monkey infections. The Serény test is based on the capacity of invasive shigellae to induce a purulent keratoconjunctivitis in guinea pigs when inoculated directly onto the conjunctiva.

S. flexneri invasiveness is encoded by a 220-kilobase plasmid. Through transposon mutagenesis and cosmid vector cloning, the invasion capability of *S. flexneri* has been localized to 31 kilobases in the pathogenicity-plasmid (Maurelli *et al.*, 1985). This 31-kilobase contains essentially the *ipa* genes (*i*nvasion *p*lasmid *a*ntigens) and the *mxi/spa* genes (*m*embrane e*x*pression of *I*pas, *s*urface *p*resentation of Ip*a*s). The *ipa* genes encode the IpaA, IpaB, IpaC and IpaD polypeptides which are the dominant antigens in the humoral response to shigellosis. Using transposon insertion and deletion mutagenesis it has been demonstrated that the *ipa*B, *ipa*C and *ipa*D genes, which belong to the same transcriptional unit, are essential for entry and vacuolar escape (Sasakawa *et al.*, 1988; High *et al.*, 1992; Ménard *et al.*, 1993). IpaB (62 kDa) and IpaC (42 kDa) are chaperoned and partitioned in the bacterial cytoplasm by IpgC, a 17 kDa protein encoded by a gene located upstream *ipa*B. Once released into the bacterial supernatant via the Mxi/Spa apparatus, the IpaB and IpaC proteins form a molecular complex which interacts with the host cell surface, thus triggering major cytoskeletal rearrangements which cause entry of the pathogen (Ménard *et al.*, 1994a). These invasion-associated proteins are maintained as a cytoplasmic pool in the bacterial cytoplasm, IpaB and IpaD forming a 'cap' which blocks their release via the Mxi/Spa apparatus. Upon contact with the host cell surface, the cap is released and the invasion-associated proteins, particularly IpaB and IpaC appear massively in the bacterial surpernatant (Ménard *et al.*, 1994b). This is an example of cross talk between a bacterium and its cellular target. Both the *mxi* and *spa* genes code for the secretion apparatus to release other plasmid-encoded proteins (Andrews *et al.*, 1991; Allaoui *et al.*,

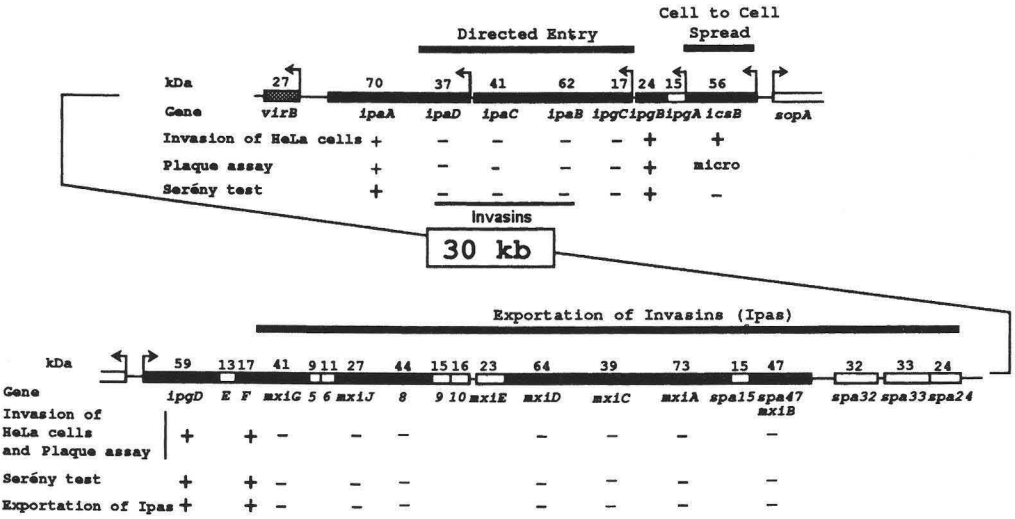

Fig. 1. Genetic map of the 30-kb entry region of *Shigella flexneri* virulence plasmid.

1992; Allaoui *et al.*, 1993). These two divergently transcribed operons are shown on Figure 1. The plasmid also contains *icsA* (*virG*), a gene essential for intracellular and intercellular movement (Bernardini *et al.*, 1989).

Intracellular movement and epithelial colonization

*Shigella*e do not have flagella and therefore are non-motile. *S. flexneri* uses the host-cell cytoskeleton to move around the cytoplasm and from cell to cell (Goldberg & Sansonetti, 1993). In infected cells, short filaments of actin form tight bundles at one pole of intracellular bacteria. The actin filaments subsequently form a tail that can be several micrometers long and contains actin-binding proteins such as plastin (Prévost *et al.*, 1992). This actin tail forms on one pole of the bacterium and makes it move as a probable consequence of filaments elongation and aggregation.

Mutants of the *icsA* gene are invasive, but once in the cytoplasm, they do not form an actin tail, do not move and cannot spread to adjacent cells (Bernardini *et al.*, 1989). IcsA is a surface protein with ATPase activity which either directly or indirectly induces actin nucleation and is localized to only one pole of the bacterium (Goldberg *et al.*, 1993). This polarity of IcsA localization provides the system with an asymetry of actin localization which is likely to allow the motor's efficiency. This protein can be phosphorylated by a cAMP-dependent protein kinase (d'Hauteville & Sansonetti, 1992), it is cleaved into a 95 kDa molecular species which is released into the bactierial supernatant.

Pathogenicity of an *icsA* mutant was tested on macaque monkeys and evaluated by endoscopy and histopathology (Sansonetti *et al.*, 1991). In contrast

to monkeys infected with the wild type parental strain, the *icsA* mutant caused only mild clinical symptoms. This mutant caused a few scattered abscesses with nodular morphology and small bloody ulcerations. In biopsies, these infectious foci showed superficial alterations of the mucosa overlaying lymphoid nodules, or in the case of ulcerations there was destruction of the mucosal surface, over lymphoid nodules, that became exposed to the colonic lumen.

The mild symptoms seen in monkeys infected with the *icsA* mutant indicate that intracellular movement, as well as cell to cell spread, play a crucial role in the development of full blown shigellosis. The fact that the few abscesses caused by this mutant localized to lymphoid nodules strongly suggests that *Shigella* penetrates the submucosa *via* M-cells.

The importance of intercellular spread in shigellosis is also examplified by the function of another pathogenicity plasmid gene: *icsB* (Allaoui *et al.*, 1992) (Figure 1). This gene is essential for promoting passage from one infected epithelial cell to the cytoplasm of the next cell. A mutant in *icsB* does not cause keratoconjunctivitis in guinea pigs, suggesting that cell to cell spread by itself is essential for pathogenicity.

Based on the reported observations, passage from cell to cell turns out to be an essential component of the epithelial colonization process by *Shigellae*. This passage causes the formation of protrusions, expansions of the membrane of the infected cell by the moving microorganism which enters the next cell. We have recently demonstrated that in the context of the colonization of an epithelial layer, protrusions are actively endocytosed by neighbouring cells and that this process involves cadherins (Sansonetti *et al.*, 1994), a family of cell adhesion molecules that are expressed essentially in the area of cells' intermediate junctions. These data allow to describe a cycle of epithelial infection and allowing extensive epithelial colonization without an extracellular phase for the bacterium. The potential efficiency of this system that protects intracellular bacteria against effector components of immune defenses is certainly reflected in the dramatic effect that the *icsA* mutation has on reducing the severity of intestinal lesions and intensity of clinical symptoms.

Bacterial effraction into the colonic mucosa

S. flexneri is only capable of invading epithelial cells through their basolateral membrane. This paradoxical observation was made in a study investigating invasion by *Shigella* of the human colonic epithelial cell line Caco-2 which establishes a confluent epithelial monolayer where cells are polarized and form an apical brush border. Therefore, the apical face of a Caco-2 monolayer mimicks the epithelial surface that a bacterium should encounter in the *in vivo* situation. When Caco-2 cells are infected with *S. flexneri*, only a few bacteria interact with the apical surface of cells, and are incapable of entering them. When the epithelial monolayer is pretreated with EGTA that disrupts the intercellular junctions by chelating Ca^{2+}, the cells become efficiently infected (Mounier *et al.*, 1992). These data suggest that *S. flexneri* cannot directly infect

epithelial cells in intact epithelia and that during a natural infection, the bacteria may have to reach the subepithelial tissues in order to enter the epithelial lining. These results are consistent with observations in electron microscopy of infected ileal loops made in rabbits. When epithelia were observed shortly after infection, there were no bacteria detectable inside epithelial cells (Perdomo *et al.*, 1994). *Shigellae* appear to be rather unique in their selectivity for the basolateral membrane. Other enteric pathogens, like *Salmonellae*, can invade cells through the apical pole (Falkow *et al.*, 1992).

M-cells are specialized cells which develop only in the epithelial dome covering the lymphoid follicles associated with the mucosa (Kraehenbühl & Neutra, 1992). These cells have the function of non-selectively transporting intact antigens from the lumen into the gut-associated lymphoid tissues (GALT) to ensure a mucosal immune response. M-cells can be differentiated from enterocytes by their characteristic morphology: shorter villi (microfolds), intense activity of endocytosis, invagination by lymphocytes and macrophages. Several enteric pathogens appear to use M-cells as a pathway to cross the epithelium. These include reovirus, poliovirus, retroviruses, bacterial pathogens such as *Salmonellae* and *Yersiniae*. M-cells also transport *Shigellae* into the lymphoid tissue (Wassef *et al.*, 1989). Observations, both in the rabbit ligated ileal loop model and in biopsies of experimentally infected monkeys indicate that the initial entry of *S. flexneri* into the mucosa is *via* M-cells (Wassef *et al.*, 1989; Perdomo *et al.*, 1994).

After translocation through M-cells, the microorganisms are delivered into the follicular structures which represents the inducive site that generates mucosal immune response (Kraehenbühl & Neutra, 1992; Liu & MacPherson, 1993; Pavli *et al.*, 1993) and also the first line of natural immune defense, essentially due to the presence of resident tissue macrophages (Soestayo *et al.*, 1990; Jarry *et al.*, 1989). Most microorganisms that encounter this cell are killed, due to the numerous antibacterial components that they produce. Pathogenic bacteria have evolved different mechanisms to survive this hostile environment. *Legionella pneumophila* and *Mycobacterium tuberculosis* inhibit fusion of lysosomes to the phagocytic vacuole. Other microorganisms, like *Coxiella burnetti*, resist the phagolysosomal environment (Falkow *et al.*, 1992). As an alternative strategy, *Shigella* avoids being killed by the macrophage by triggering the phagocyte's suicide program (Zychlinsky *et al.*, 1992).

S. flexneri induces apoptosis of macrophages

Necrosis and apoptosis are the two major forms of cell death. Necrosis is a passive process, which occurs when a cell dies because of physical alteration. Apoptosis, or programmed cell death, needs active participation of the cell. Apoptotic cell death has been described during normal embryonic development and differentiation, in tumor regression and during growth factor deprivation (Arends & Wyllie, 1991; Ellis *et al.*, 1991).

We have reported that the mechanism of cytotoxicity by which *S. flexneri* kills macrophages (Clerc *et al.*, 1987) is induction of programmed cell death (Zychlinsky *et al.*, 1992). Apoptosis is induced when either a membrane or a cytoplasmic receptor binds an appropriate ligand which induces expression of a second messenger (Arends & Wyllie, 1991; Ellis *et al.*, 1991). An increase in the concentration of intracellular calcium (Caron-Leslie & Cidlowski, 1991; Ojcius *et al.*, 1991) and cAMP (McConkey *et al.*, 1993; Vintermyr *et al.*, 1993) have been implicated, thus inducing expression of the genes necessary for cell death. There are few eukaryotic genes known to be involved in apoptosis. Expression of the tumor suppressor gene *p53* is apparently sufficient to commit a cell to undergo apoptosis. Expression of the oncogenes *c-myc* and *c-fos* is also required, but is not sufficient for the induction of apoptosis. On the other hand, the oncogene *bcl-2* can block the process of programmed cell death (Freeman *et al.*, 1993). *Shigella* only kills its host-cell if it escapes from the phagolysosome into its cytoplasm. Further investigations will hopefully allow complete understanding of the molecular bases of *Shigella*'s relationship to the macrophage (Zychlinsky *et al.*, 1993). IpaB, one of the *Shigella* invasins, accounts for macrophage death (Zychlinsky *et al.*, 1994a).

In addition to their microbicidal activity, macrophages have a crucial role in signalling to other cells the presence of an invasive agent and the generation of inflammation (Dinarello, 1992). In response to a variety of stimuli, macrophages can express three of the most important inflammatory cytokines: Interleukin-1 (IL1), IL-6 and TNFα. IL-6 and TNFα are transcribed, translated and secreted as soon as the cell is stimulated, while IL-1 is in part accumulated in the cytosol. The two forms of IL-1, IL-1α and IL-1β, bind to the same receptors and have similar biological activities. Both forms are synthesized as precursors of 35 kDa which are subsequently proteolytically cleaved by specific proteases to yield mature forms of 17.5 kDa. Both the precursors and the mature form of IL1α and only the mature form of IL-1β are biologically active. The precursor forms of IL-1 are synthesized and accumulated in the cytosol of macrophages. The mature forms are found exclusively in tissue culture supernatants. In the absence of a secretion signal sequence, IL-1 molecules are not released *via* a classic secretory pathway (Dinarello, 1992). It has been shown that one of the possible mechanisms for this cytokine release is apoptosis (Hogquist *et al.*, 1991).

In order to confirm that macrophage apoptosis may play a significant role in the initiation of inflammation, we studied the release of inflammaroty cytokines by infected macrophages *in vitro*. We tested cytokine release by LPS-stimulated murine peritoneal macrophages infected with *S. flexneri*. These cells contain pools of IL-1 and may reflect the activation state of colonic macrophages (Youngman *et al.*, 1993; Mahida *et al.*, 1989; Mahida *et al.*, 1989; Soestayo *et al.*, 1990; Jarry *et al.*, 1989) which are likely to be permanently exposed to bacterial products originating from the intestinal flora. Large amounts of IL-1, but not IL-6 or TNFα, are released by stimulated peritoneal macrophages infected with wild types *S. flexneri*, but not with a non-invasive derivative. Time course experiments of cytokine release demonstrate that IL-1 is present in the super-

nant of infected cells as early as 15 minutes after infection. IL-1 release asssayed either by its biological activity or antigenically in an ELISA assay, happens before macrophage integrity is compromised. The supernants of LPS-stimulated macrophages infected with *Shigella* contain IL-1α in its active precursor form and IL-1β both in its precursor and mature forms. These results suggest that release of IL-1 happens early in apoptosis and that the release is an active process and not the result of leakage of intracellular stores of cytokines when the integrity of the plasma membrane is compromised (Zychlinsky *et al.*, 1994b).

Bacterial-induced apoptosis of macrophages might play an active role *in vivo* by eliciting an early inflammatory response in epithelial tissues. Release of IL-1 would be a primary, if not the first signal to initiate the inflammatory process. This signal will subsequently be amplified by the induction of the production of other inflammatory cytokines such as IL-6, IL-8 and TNFα by other cells: non-infected macrophages, lymphocytes, endothelial and epithelial cells (Agace *et al.*, 1993; Nakamura *et al.*, 1991).

PMN leukocytes facilitate *S. flexneri* invasion

A major characteristic of shigellosis is the extensive inflammatory reaction and tissue destruction found far beyond Peyer's patches in the rabbit ligated ileal loop model and lymphoid follicles in biopsies of humans and monkeys (Anand *et al.*, 1986; Sansonetti *et al.*, 1991). In these late stages numerous bacteria are found inside epithelial cells. These data suggest that other secondary sites of bacterial entry may exist besides M-cells or that lymphoid follicles and associated dome epithelium are the center of a centrifugal inflammation that extends progressively away from the initial site of bacterial entry.

We hypothesized that the early inflammatory reaction induced by the release of IL-1 by apoptotic macrophages may provoke edema and extravasation of PMN cells. PMN cells (Dinarello, 1992) have a strong chemotactic activity toward gram-negative bacteria and migrate to the lumen of the colon. The transmigration of PMN cells would destabilize and eventually destroy the epithelial barrier, allowing access to the basolateral membrane of colonocytes and their subsequent invasion. Data *in vitro* strongly support this model (Perdomo *et al.*, 1994). The migration of PMN leukocytes was tested using the cell line T-84. This human colonic cell line is grown on permeable supports, the cells become polarized, they present microvilli on their apical side and develop junctions (McRoberts & Barrett, 1989). PMN leukocytes migrate through the epithelium in response to the presence of *Shigella* on the apical side of colonocytes. This migration disrupts intercellular junctions and opens the paracellular pathway for bacteria to reach the basolateral side of epithelial cells and to invade. The structure of the T-84 epithelial lining is reminiscent of intestinal crypts. This system has been used to study chemotactic substances that induce the transmigration of neutrophils across epithelia (Colgan *et al.*, 1993).

When PMN leukocytes are placed on the basolateral side of the epithelium and bacteria on the apical side, both the wild-type strain of *S. flexneri* and a

plasmid-cured, non-pathogenic derivative provoke efficient transmigration of neutrophils across a 3-day old T-84 monolayer. However, only the wild-type strains efficiently invade the epithelial cells. In control experiments, where only bacteria were added to the apical side without PMN cells in the system, there was no significant invasion of T-84 cells as previously mentioned in this review. These results indicate that *S. flexneri*, like other enterobacteriaceae, can induce the transmigration of PMN cells and that destruction of the epithelial barrier provides bacteria an access to the basolateral membrane cells. Release of oxygen radicals as well as proteolytic enzymes, like elastase, metallo-proteases and serine proteases, by PMN cells could destroy the epithelium through an 'innocent by-stander' effect, providing even greater access for bacteria to infect the basolateral membrane of epithelial cells (Weiss, 1989).

Transmigration requires specific adhesion of PMNs to epithelial cells. The interaction between these two cells is essentially mediated by the integrin CD11b/CD18 (Mac 1) (Parkos *et al.*, 1991). Monoclonal antibodies against CD18 can neutralize PMN cell transmigration, as well as consequent epithelial cell invasion. The importance of PMN leukocytes in the invasion of *Shigella* has recently been confirmed in experiments carried out in the rabbit ligated ileal loop assay. In these experiments (Perdomo *et al.*, 1994) rabbits have been preloaded with a monoclonal antibody against CD18, before ligating the ileal loops and infecting them with wild-type *Shigella*. Under these conditions a very strong inhibition of tissue inflammation was observed, and almost no bacterial invasion could be detected. These results confirm that bacterial passage through the epithelial barrier depends very much on transmigration of PMN cells and consequent destruction of epithelial cell junctions.

Conclusion

Taking together information from tissue cultured cells, animal models and histopathology from experimentally infected monkeys and patients, we propose here a model for the initiation of shigellosis. Figure 2 shows the proposed sequence of events. First, *S. flexneri* makes contact with M-cells in the colon and is transcytosed from the lumen into the mucosa. M-cells deliver bacteria directly into the dome of lymphoid follicles. These areas are densely populated by resident tissue macrophages, which are infected by *Shigella*. Infection induces macrophage apoptosis, with the concomitant release of IL-1. *Shigella* then starts disseminating through the epithelium and the *lamina propria*. IL-1 released by macrophages starts an inflammatory response. Consecutive extravasation of PMN leukocytes from the circulation into the submucosa, followed by chemoattraction into the lumen of the colon, disrupts the integrity of the epithelial barrier. *S. flexneri* massively infects epithelial cells through their basolateral membrane. Bacterial products induce the production of more cytokines which potentiate inflammation. In the final stage, major destruction of the epithelium is observed. This process resembles certain aspects of acute inflammatory bowel

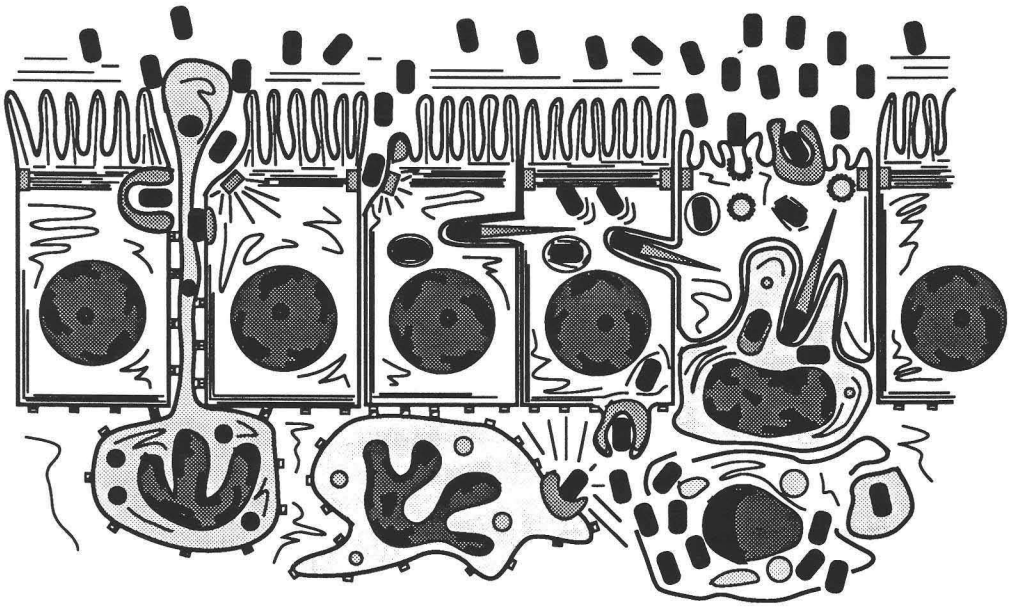

Fig. 2. Scheme of the global process of invasion of the epithelial barrier by *Shigella flexneri.* Initial event involving entry into M-cells, phagocytosis by local macrophages in the vicinity of lymphoid follicles, induction of macrophages apoptotic death with release of inflammatory cytokines. Subsequent events involving invasion of the *lamina propria* by polymorphonuclear cells (PMN), destabilization of epithelial layer by PMN transmigrating through, under chemoattractant potential of luminal bacteria, entry through basolateral side of epithelial cell and development of the colonization process by cell to cell spread.

diseases such as ulcerative colitis. It must be considered, especially for future vaccine development.

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