# 1. INTRODUCTION

## 1.1. The Intestinal Mucosa and Local Immune System

The main function of the intestinal tract is to absorb nutrients. This is accomplished by a monolayer of absorptive columnar epithelial cells, which exhibit long microvilli on their apical surface covered with a thick mesh of glycoproteins and glycolipids (the glycocalyx) and further covered by thick mucus. Due to the microvilli, villi and crypts the total exchange area between gut lumen and mucosa is approximately 100 $m^2$ (Pabst, 1987), which is about 60 times larger than the surface area of the skin. However, the epithelial cells provide a somewhat fragile boundary between the underlying mucosal tissue and the lumen rich in nutrients, foreign antigens, flora (microbiota) and sometimes pathogens. Hence, the epithelium must be permeable to electrolytes and nutrients, yet provide an effective barrier function against harmful macromolecules and organisms. As well as the barrier and absorptive functions the intestinal epithelium must provide effective local immunity against pathogens and their toxins, but at the same time tolerate the normal flora.

The ability to distinguish between a potentially harmful pathogen or toxin and an antigen that should be tolerated, is mediated by a specialised local immune system termed ‘gut associated lymphoid tissue’ (GALT). The GALT has two functional components: i) an inductor part consisting of the organised lymphoid follicles, eg Peyer’s patches, and ii) an effector part consisting of the lymphocytes scattered throughout the lamina propria and those found above the basement membrane, ie intra-epithelial lymphocytes (IEL). The detailed structure of the GALT is as follows: it should be noted that similar structures, with identical functions, are also to be found in the respiratory system (Claeys et al, 1996a, Claeys et al, 1996b).

## 1.2. Lymphoid Follicles and Follicle Associated Epithelium

The organised lymphoid follicles are an extremely important part of the GALT and are responsible for much of the induced immune response to foreign

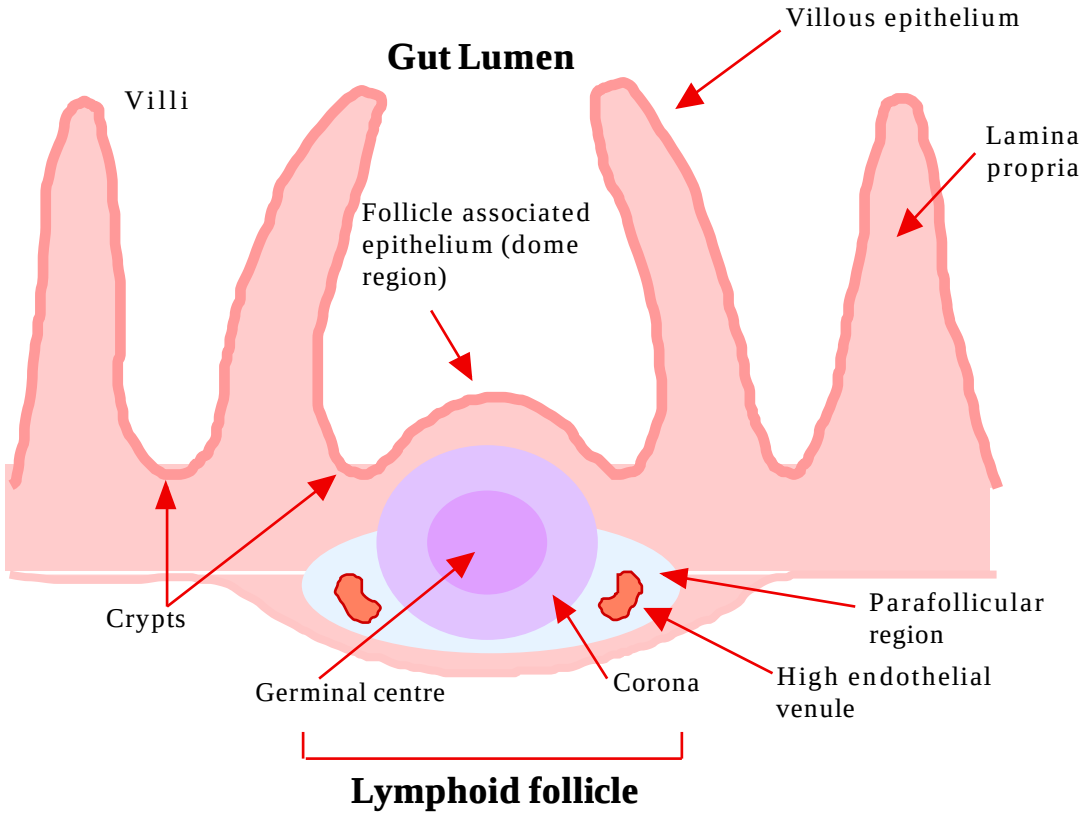


**Fig. 1.1.** Diagram showing the structure of an intestinal lymphoid follicle. Lymphoid follicles lying in the submucosa are associated with dome areas that bulge into the gut lumen. The dome areas are covered with a specialised epithelium, characterised by the presence of M cells. While B lymphocytes predominate in the lymphoid follicles, the parafollicular region is populated mainly by T lymphocytes.

antigen. Each lymphoid follicle contains a variety of cell types organised in four distinct structures (see Fig. 1.1):

i) Proliferating B lymphocytes form germinal centres which also contain macrophages that phagocytose the remnants of apoptosed lymphocytes. The majority of B lymphocytes are preprogrammed to mature into IgA producing plasma cells (Köhne et al, 1996).

ii) Each follicle is surrounded by a corona of small lymphocytes, many of which express IgM and IgD on their cell surface.

iii) The interfollicular areas are populated by high endothelial venules (blood vessels) and lymphatic microvessels, both surrounded by densely packed lymphocytes (predominantly T cells). This is where lymphocytes enter the follicles from the blood, and lymphocytes leave via the lymphatics.

iv) Just below and within the epithelium overlying the follicle, a mixture of

lymphocyte populations can be found.

In total, each follicle comprises mainly B cells (~60 %) and T cells (~40 %), with macrophages and dendritic cells incorporating ~0.4 % (MacDonald and Carter, 1982). Unlike the spleen and lymph nodes the GALT contains no capsule surrounding the follicles. Aggregates of lymphoid follicles in the wall of the small intestine are termed Peyer's patches after their founder Peyer (in 1677).

To facilitate antigen sampling the lymphoid follicles of the GALT have a specialised epithelium overlying the follicle called 'follicle associated epithelium' (FAE). The FAE has certain important characteristics which make it significantly, and functionally, different from the epithelium found overlying the villi structures (reviewed in Gebert et al, 1996): firstly, the FAE contains almost no mucus producing goblet cells; secondly, the FAE exhibits reduced expression of genes required for the digestion and transport of nutrients; thirdly, transport of polymeric immunoglobulins is significantly diminished in the FAE due to the total absence of polymeric immunoglobulin receptors; and lastly, the FAE contains M cells.

M cells (microfolded or membranous epithelial cells) are the most radical adaptation of epithelial cells by the FAE to promote selective entry of enteric antigens into the lymphoid follicle for antigen processing and presentation (see Fig. 1.2). Morphologically, M cells exhibit shorter and more widely spaced microvilli, a flexible cytoskeleton facilitating transcytosis of particles, are highly visiculated due to increased transcytosis, and they often have immune cells in a pocket formed in the basolateral membrane (Savidge, 1996, Gebert et al, 1996). It is also important to note that the glycocalyx overlying M cells is considerably thinner than that overlying adjacent columnar epithelial cells (Savidge, 1996).

M cells express specific cell surface markers that both help to identify them in scientific studies and, are very likely to be important in adherence of antigen before triggered endocytosis. The polysaccharides of membrane bound glycoproteins and glycolipids are one such marker group, whereby a particle with a lectin like structure, recognising the expressed glycoprotein or glycolipid on the

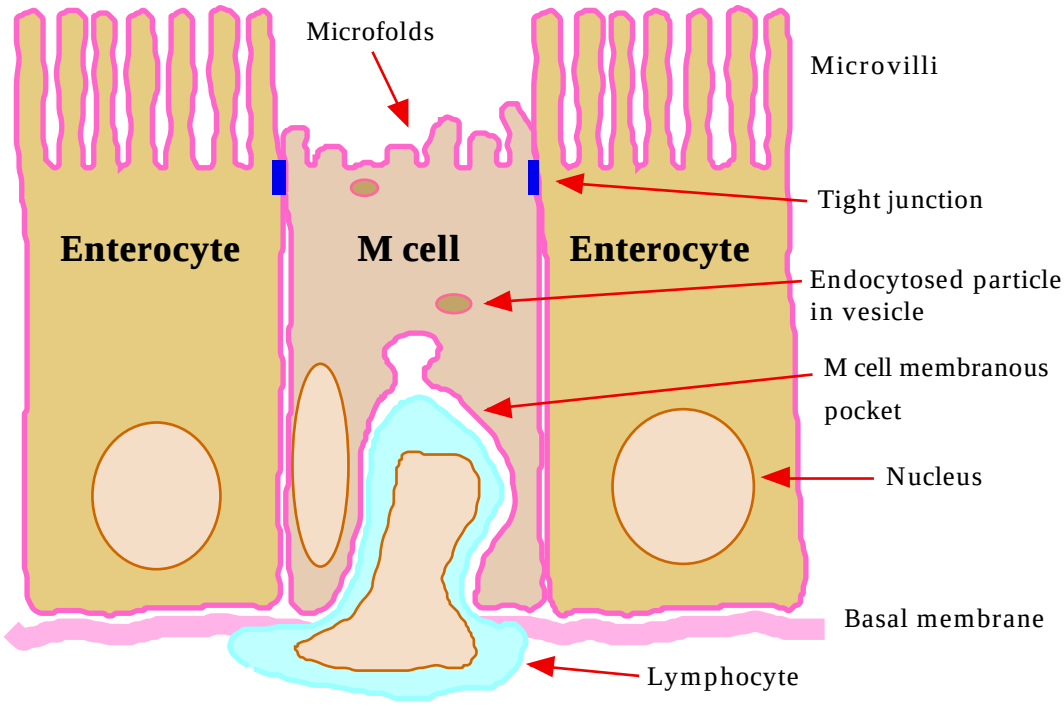


**Fig. 1.2.** Diagram showing an M cell between two enterocytes within the FAE of a dome area. Antigen uptake by M cells is followed by transcytosis and exocytosis into the M cell pocket. Lymphocytes are the common cell types found in the pocket (macrophages and dendritic cells may also be found), and immediately take up the exocytosed antigen for processing and presentation to the underlying lymphocytes. Paracellular transport of particles is hindered by the presence of tight junctions.

M cell surface, could intimately associate with the plasma membrane. In murine and rabbit FAE the agglutinin of *Ulex europaeus*-1 (UEA-1) specifically recognises M cells of the small intestine (Clark et al, 1993) by binding to fucose residues and thus UEA-1 coupled to a fluorescent dye is often used as an M cell marker for fluorescent microscopy studies. Unfortunately, no human M cell marker has been found that is as specific as UEA-1 for murine M cells.

Together these attributes encourage close interaction of luminal particles with M cells due to less mucus and thinner glycocalyx, reduce local immune exclusion from sIgA agglutination, and aid unaltered (non-digested) antigen arriving within the follicle.

It has also been shown that oral inoculation of germ free mice with *Salmonella typhimurium aroA*$^{-}$ stimulates the GALT, resulting in higher numbers of M cells within the murine FAE (Savidge et al, 1991). Hence, the M cell population can increase upon demand, increasing the chance of a pathogen or toxic molecule

being sampled, and that in turn increasing the chance of an effective immune response.

### 1.3. Interaction of Antigens at the M Cell Surface and Induction of an Immune Response

It has been demonstrated that transcytosis of antigens by M cells is a prerequisite for the induction of a successful immune response (Neutra et al, 1987). M cells use multiple endocytic mechanisms for uptake of macromolecules, particles and microorganisms. Macromolecules, including viruses, are endocytosed via clathrin coated pits and vesicles (Neutra et al, 1987). non-adherent materials are taken up by fluid phase endocytosis in coated or non-coated vesicles (Bockman and Cooper, 1973, Owen, 1977). Larger adherent particles, eg bacteria, trigger phagocytosis involving membrane rearrangement through actin cytoskeletal reorganisation. Once the antigen has been endocytosed or phagocytosed it is quickly transported to a tubulovesicular system called the endosomal compartment, and then released into (exocytosis) the basal membrane pocket of the M cell (Neutra et al, 1987). This whole process typically takes between 10-15 min. Interestingly, in M cells the endosomes bypass lysosome fusion thereby releasing the antigen without enzymatic degradation having occurred (Owen et al, 1986b).

Within the M cell pocket are B and T lymphocytes (more $CD4^+$ than $CD8^+$), macrophages, or occasionally, dendritic cells awaiting antigen. Antigen is taken up by these cells, processed and presented to trigger an immune response. Due to the cell population in the FAE and follicle the immune reaction is biased toward a CD4 mediated antibody response, but a CD8 response is also possible. After initiation of the immune reaction, primed B lymphocytes and lymphoblasts migrate as precursors of IgA secreting plasma cells via the intestinal lymphatics, mesenteric lymph nodes, thoracic duct and peripheral blood to the lamina propria of the gut. However, due to the homing molecules expressed on these primed cells they not only home to the gut, but also to other mucosal sites, eg the

respiratory tract and the genital tract in females, although the immune response is frequently stronger where the initial stimulation occurred (Husband and Gowans, 1978).

## 1.4. Bacterial Interaction with M Cells

Bacteria that cause disease in the intestinal tract may be categorised into those that primarily produce toxins (Finlay and Falkow, 1997), eg *Vibrio cholerae* and *Clostridia difficile,* and those that invade intestinal cells (Finlay and Falkow, 1997), eg *Shigella flexneri,* although these two categories are not mutually exclusive. Another category of bacterial pathogens may be described as those that enter the host organism via the intestine and cause a systemic disease, eg *Listeria monocytogenes* (reviewed in Farber and Peterkin, 1991) and *Salmonella typhi* (Mandal, 1994). It has become increasingly apparent that many bacteria that cross the epithelial barrier preferentially infect, or associate with, M cells in Peyer's patches (see Table 1.1). Invasion of M cells is sometimes part of the pathogenic process undertaken by the bacteria resulting in disease, but sometimes seems to

| Organism | Host | Reference |
|---|---|---|
| **Bacteria** | | |
| Bacillus Calmette-Guérin | Rabbit | Fujimura, 1986 |
| *Brucella abortus* | Cattle | Ackerman et al, 1988 |
| *Campylobacter jejuni* | Rabbit | Walker et al, 1988 |
| *Escherichia coli (RDEC-1)* | Rabbit | Inman and Cantey, 1983 |
| *Mycobacterium paratuberculosis* | Cow | Momotani et al, 1988 |
| *Salmonella typhi* | Mouse | Kohbata et al, 1986 |
| *Salmonella typhimurium* | Mouse | Jones et al, 1994 & Clark et al, 1994 |
| *Shigella flexneri* | Rabbit | Wassef et al, 1989 |
| *Streptococcus pneumoniae* | Rabbit | Regoli et al, 1995 |
| *Vibrio cholerae* | Rabbit, Man | Owen et al, 1986a |
| *Yersinia enterocoloitica* | Mouse | Hanski et al, 1989 |
| *Yersinia pseudotuberculosis* | Rabbit | Fujimura et al, 1989 |
| **Viruses** | | |
| HIV | Mouse | Amerongen et al, 1991 |
| Mouse Mammary Tumour Virus | Mouse | Neutra and Kraehenbuhl, 1992 |
| Poliovirus | Man | Sicinski et al, 1991 |
| Reovirus | Mouse | Wolf et al, 1981 |
| **Other Microorganisms** | | |
| Baker's Yeast | Pig | Gebert et al, 1994 |
| Cryptosporidium | Guinea pig | Marcial and Madara, 1986 |
| *Giardia muris* | Mouse | Owen et al, 1979 |

**Table 1.1.** Uptake of microorganisms by M cells of the Peyer's patch FAE in different animal species.

give no advantage to the bacteria whatsoever. For example, *Shigella flexneri* specifically attaches to and invades M cells of the rabbit small intestine, but is unable to invade differentiated columnar epithelial cells from the apical side (Perdomo et al, 1994). After invasion the bacteria escape rapidly from the M cell vacuole, replicate, and form actin tails which propel the bacteria into neighbouring cells. Infected macrophages undergo bacterial induced apoptosis with accompanying cytokine release. This induces a massive influx of inflammatory cells which destroys the tight monolayer formed by the enterocytes. In contrast to *Shigella flexneri*, *V. cholerae* normally adheres to some particular unidentified component of the glycocalyx on microvilli tips of columnar epithelial cells, but does not enter these cells, allowing almost direct action of cholera toxin. But when *V. cholerae* is in close proximity to M cells the bacteria are quickly taken up and processed for antigen presentation (Owen et al, 1986a). The resulting sIgA response can be enough to clear the infection and prevent subsequent colonisation (Levine et al, 1988).

The subject of this work pertains to two bacteria and their interaction with intestinal epithelial cells, namely *S. typhimurium* and *L. monocytogenes*. These two organisms are now discussed in detail with respect to their adhesion, invasion and post invasive actions.

## 1.5. The Gram Negative Pathogen *Salmonella typhimurium*

*Salmonella enterica* serovar Typhimurium (hereafter called *S. typhimurium*) is a food borne pathogen which causes gastroenteritis in humans and is one of the most commonly isolated bacteria from patients with symptoms of gastroenteritis in the UK (see Table 1.2). Interestingly, the same bacteria causes a systemic, typhoid like disease in mice. *S. typhi* is the aetiological agent of typhoid in humans, but has a very narrow host specificity which does not allow the study of this organism in mice. Hence, *S. typhimurium* infected mice are often used as an enteric fever infection model. However, under specific conditions *S. typhi* does

| Year | *Campylobacter spp.* | *S. typhimurium* | *S. enteritidis* |
|---|---|---|---|
| 1986 | 24809 | 7094 | 7742 |
| 1987 | 27310 | 7660 | 11820 |
| 1988 | 28761 | 6444 | 27949 |
| 1989 | 32526 | 7306 | 28704 |
| 1990 | 34552 | 5451 | 34991 |
| 1991 | 32636 | 5331 | 32153 |
| 1992 | 38552 | 5401 | 37081 |
| 1993 | 39422 | 4778 | 37511 |
| 1994 | 44414 | 5522 | 31153 |
| 1995 | 43876 | 6743 | 28526 |
| 1996 | 43337 | 5542 | 31383 |
| 1997 | 50201 | 4778 | 38274 |
| **Total** | **440396** | **72050** | **347287** |

**Table 1.2.** The top three causes of infectious intestinal disease in England and Wales, 1986-1997. Although there are more individual cases of *Campylobacter* spp. than *Salmonella* spp., *Salmonellae* are more often associated with outbreaks. Source: UK Communicable Diseas Surveillance Centre web site (http://www.phls.co.uk/).

interact with murine FAE in a similar fashion to *S. typhimurium* (Kohbata et al, 1986, also see later). *S. typhimurium* is one of the most researched human pathogens due to its relatively easy genetic manipulation and a reproducible, cheap animal infection model. It is important to note that almost all in vitro and in vivo research revolves around the ability of *S. typhimurium* and *S. typhi* to cause enteric fever in the mouse and man, respectively. How *S. typhimurium* causes diarrhoea in man is largely unknown.

### 1.5.1. Invasion of Cultured Epithelial Cells by *S. typhimurium*

*S. typhimurium* triggers a dramatic response from the epithelial cell surface shortly after contact with the host cell. The host cell surface extrudes outward from the point of bacterial adherence with attendant localised membrane ruffling and macropinocytosis (Francis et al, 1993), which is very similar to an already known phenomenon-in eukaryotic cells seen after certain signal transduction events (Ridley, 1994). Additionally, actin filaments are rearranged and host surface proteins are capped, but host actin and the cell surface return to normal after the bacteria are internalised (Finlay et al, 1991). Several signal transduction pathways, including calcium and inositol phosphate fluxes, are activated within

the host cell (Pace et al, 1993, Ruschkowski et al, 1992), although it is not known if these pathways are activated to initiate invasion, or because of invasion. As to the receptor(s) that *S. typhimurium* activates, little is known. An appealing idea was recently published that indicated the epidermal growth factor receptor was activated by *S. typhimurium* resulting in ruffling and uptake (Galán et al, 1992, Pace et al, 1993). However, other published works have since demonstrated that the EGF receptor is either only indirectly involved in invasion, or not at all, both in vitro (Jones et al, 1993, Rosenshine et al, 1994) and in vivo (McNeil et al, 1995). Central to the regulation of the cytoskeleton are members of the Ras related superfamilies of small GTPases, Rac, Rho, and CDC42 (Nobes and Hall, 1995), which coordinate the formation of actin based structures involved in cell motility, cytokinesis, phagocytosis, and intracellular transport processes. However, although it seems likely that *S. typhimurium* somehow interferes with cytoskeleton regulation, bacterial entry has been found to be Rac and Rho independent (Jones et al, 1993). Nevertheless, early data indicates that CDC42 is a favourable candidate (Chen et al, 1996b).

*S. typhimurium* has multiple genetic loci involved in virulence, including at least two discrete pathogenicity islands, SPI1 (reviewed in Galán, 1996b) and the more recently described SPI2 (Hensel et al, 1995, Hensel et al, 1997). SPI1 and SPI2 both encode distinct and independent type III secretion systems also containing their secreted targets, chaperones and regulators (reviewed in Hueck, 1998). Furthermore, cross talk between the two islands has been shown to be likely, in that SPI2 could be involved in secreting the targets of SPI1 (Hensel et al, 1997). The exact role of SPI1 and SPI2 in invasion is still unclear. Recent results indicate that *S. typhimurium* assembles filamentous surface appendages upon contact with epithelial cells which disappear just before entry. These appendages are missing in bacteria grown in the absence of cells. When certain mutations are introduced into some of the *inv* genes in SPI1 two phenotypes are observed: either surface appendages are never lost (*invA* and *invE*), or they are never formed (*invC* and *invG*), but all four of these mutations induce a non-invasive

phenotype (Ginocchio et al, 1994). However, the work of Reed et al (1998) somewhat contradicts these data as they report that non-invasive mutants (mutations in the SPI1 loci) always build appendages upon contact with epithelial cells in vitro and in vivo. Moreover, they report that a PhoP constitutive mutant rarely made the appendages, although the effect on invasion efficiency was not investigated.

### 1.5.2. Invasion of Epithelial Cells by *S. typhimurium* In Vivo

*S. typhimurium* infection of the murine host can be divided into two distinct stages: crossing of the intestinal epithelial barrier, and subsequent survival in the lymphatic system. The first indication that a particular part of the small intestine was important for *Salmonella* spp. crossing the epithelial barrier was shown by Carter & Collins (1974). They showed the strong preference *S. enteritidis* has for Peyer's patches after oral infection of mice. Some years later it was shown by two independent groups (Jones et al, 1994, Clark et al, 1994) that *S. typhimurium* preferentially attaches to, and invades, murine M cells of Peyer's patches by a bacterial driven process that induces massive M cell membrane rearrangement (ruffling) resulting in uptake (see Fig. 1.3), and is similar to the events observed in vitro (Francis et al, 1993). The long polar fimbriae (*lpf*) operon is involved in targeting the pathogen to murine Peyer's patches and may mediate specific adherence to M cells (Bäumler et al, 1996). These fimbriae are not the same as the appendages described earlier (Ginocchio et al, 1994) and the operon is located on a different part of the chromosome (78 centisomes) to SPI1 (63 centisomes) and SPI2 (30 centisomes). When a mutation was introduced in *lpfC* the mutant showed reduced numbers in organs of a mouse after oral infection, but not after intraperitoneal infection, suggesting a role in intestinal invasion. Furthermore, when the *lpf* operon was introduced into *Escherichia coli* the strain showed an increased ability to attach to histological thin sections of Peyer's patch tissue.

Another indirect mechanism *S. typhimurium* has for crossing the epithelial barrier is through stimulating epithelial cells to produce chemotactic cytokines

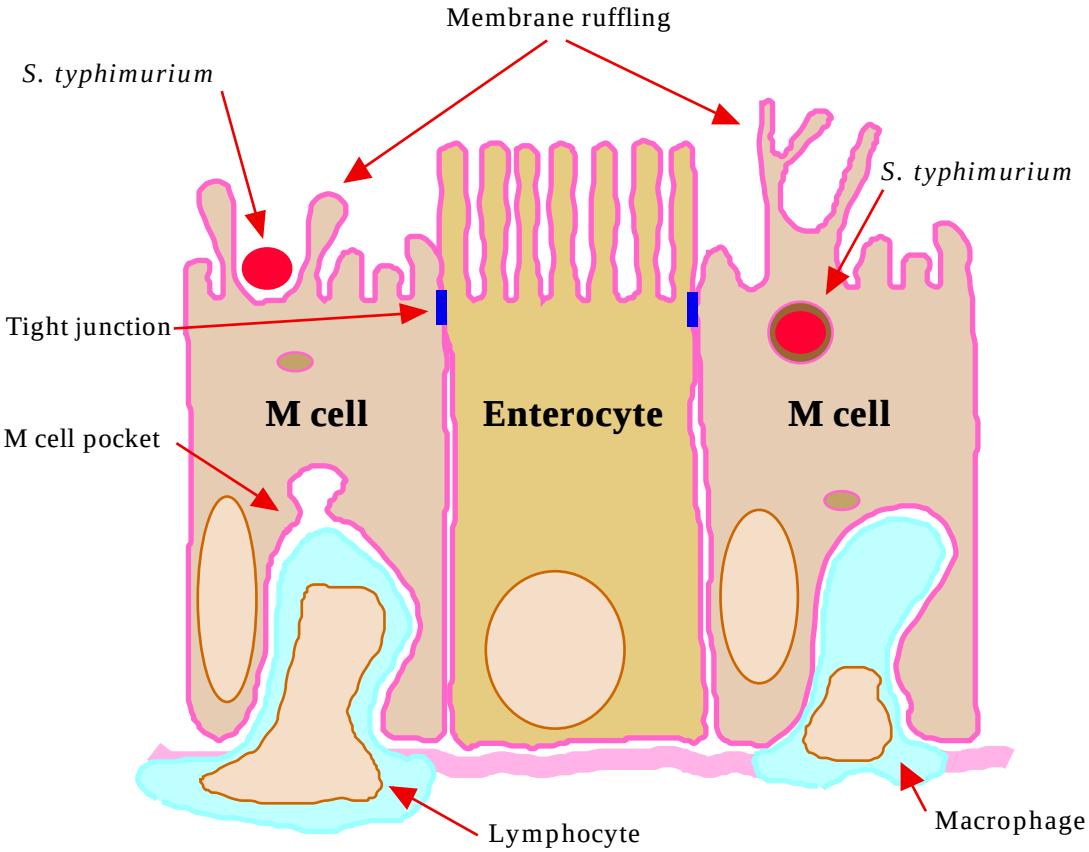


**Fig. 1.3.** Diagram showing the effect of *S. typhimurium* on M cells of the FAE. After contact the bacteria induce self uptake by triggering massive actin reorganisation yielding the distinctive membrane ruffling which accompanies invasion.

(McCormick et al, 1993, Jung et al, 1995), eg interleukin 8 (IL-8). IL-8 stimulates polymorphonuclear neutrophil (PMN) transmigration through the epithelial barrier, opening epithelial cell tight junctions hence, allowing free access for bacteria to the basolateral side. This mechanism may also be important in gastroenteritis induction by *S. typhimurium* in man (McCormick et al, 1995) and is probably the mechanism by which *Shigella flexneri* causes the clinical signs and symptoms of dysentery (Perdomo et al, 1994).

### 1.5.3. Survival of *S. typhimurium* in Deeper Tissues

As previously mentioned, *S. typhimurium* causes a systemic disease in mice with many of the clinical symptoms being attributable to LPS and therefore after it has crossed the intestinal barrier its journey has only just begun. It has been commonly thought that *S. typhimurium* invades macrophages, probably immediately after invasion of the FAE, and circulates the body via the lymphatic

system and blood until it reaches the liver and spleen, whereupon it enters the fixed resident macrophages of the respective organs, multiplies and induces disease (reviewed in Jones and Falkow, 1996). Therefore *S. typhimurium* has been thought of as a facultative intracellular pathogen. However, this assumption was based on the ability of *S. typhimurium* to survive in cultured macrophages, but direct evidence to refute or confirm this notion for the in vivo situation was, until recently, lacking. Richter-Dahlfors et al (1997) have elegantly shown, using low doses that better represent a "natural" infection, that *S. typhimurium* reside in macrophages (both the resident kupffer cells and infiltrating macrophages) in the liver until the bacteria overwhelm the host and spread to the interstitial spaces. The genes required for this process are not known, but it is likely that the genes, already identified, that give *S. typhimurium* the ability to invade (*inv* and *sip* genes- reviewed in Galán, 1996b and Galán, 1996a), survive (PhoP / PhoQ regulon- reviewed in Miller, 1991) and induce apoptosis (probably the invasion associated genes, refs Monack et al, 1996, Chen et al, 1996a, and Richter-Dahlfors et al, 1997) in cultured macrophages are either mostly, or all, required for the in vivo setting (reviewed in Jones and Falkow, 1996). This conjecture is supported by the fact that the genes found to be important in in vitro studies are often necessary for causing disease in the mouse.

Libby et al (1994) recently described a novel gene of *S. typhimurium*, termed *slyA*, which when introduced into *Escherichia coli* in multiple copies produces a haemolytic phenotype. This gene was therefore thought to encode a cytolytic toxin of *S. typhimurium* that was tentatively designated "salmolysin". The amino acid sequence of the SlyA protein deduced from the nucleotide sequence of the *slyA* gene showed, however, no sequence homology with other known bacterial cytolysins. Moreover, the possibility that SlyA could regulate a cryptic haemolysin gene in *E. coli* was not completely excluded. Recently Dehoux and Cossart (1995) reported that, after performing a more up to date homology search, SlyA shared significant sequence homology with certain bacterial global regulatory proteins, such as MprA of *E. coli* (Castillo et al, 1990, Castillo et al,

1991). Recent work has shown (Ludwig et al, 1995, Oscarsson et al, 1996) indeed that *slyA* encodes a regulatory protein that induces a cytolysin, *clyA*, when over expressed in *E. coli*. Furthermore, given that an insertion mutation in *slyA* reduces the in vivo virulence of *S. typhimurium* but this strain shows no physiological or morphological differences in vitro (Libby et al, 1994, Buchmeier et al, 1997), it is likely that SlyA regulates some virulence factor(s) of *S. typhimurium*.

## 1.6. The Gram Positive Pathogen *Listeria monocytogenes*

*L. monocytogenes* causes listeriosis in susceptible humans after ingestion of contaminated food (Farber and Peterkin, 1991, Dalton et al, 1997). However, unlike *S. typhimurium*, *L. monocytogenes* does not typically cause gastroenteritis but rather a systemic disease that may lead to meningo-encephalitis, or infection of the fetus in pregnant women (Farber and Peterkin, 1991). Of the cases of listeriosis in England and Wales between 1983-1997 32 % were associated with pregnancy, indicating this group as a notably "high risk" one (see Fig. 1.4). This may be due to a shift in the balance of T-helper 1 (Th1) and Th2 cells during pregnancy, in that Th1-type cytokines are incompatible with successful pregnancy, resulting in a host induced Th2 bias (Raghupathy, 1997). A Th1-type response is, nevertheless, necessary to clear intracellular pathogens, eg *L. monocytogenes*

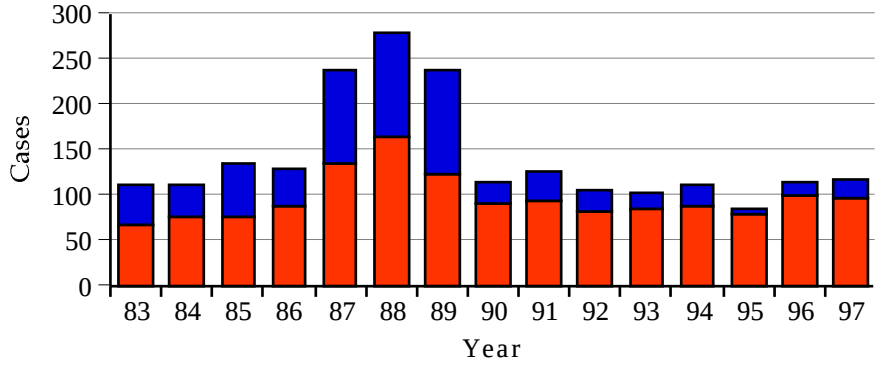


**Fig. 1.4.** Number of human listeriosis cases in England and Wales, 1983 - 1997. **Red bars** represent non-pregnancy-associated cases, **blue bars** represen pregnancy-associated cases. Note: mothers and babies are counted as one case. Source: UK Communicable Disease Surveillance Centre web si (http://www.phls.co.uk/).

(Kaufmann, 1993). Therefore, in maintaining a Th2 bias the mother nurtures her growing fetus, but is more vulnerable to intracellular infections. However, if the mother becomes infected this will stimulate a Th1-driven response that will in turn endanger the fetus, possibly resulting in fetal resorption during early pregnancy or spontaneous abortion in later pregnancy. This may play a role in the spontaneous abortions seen in pregnant humans and animals infected with *L. monocytogenes.*

Murine listeriosis was introduced 35 years ago as a model with which to analyse mechanisms of antibacterial defence that are independent of antibodies (Mackaness, 1964). This model was a parenteral infection model, ie intravenously or intraperitoneally infected mice, and so did not reflect the natural route of infection, namely through the gut. MacDonald & Carter (1980) further developed the model and showed that mice could be reproducibly infected via the oral route, and that *L. monocytogenes* associated with the Peyer's patches rather than non-Peyer's patch gut tissue. What was not investigated in this study was the question of 'why the association with Peyer's patches?'. It was, however, later assumed that *L. monocytogenes* probably crossed the intestinal epithelial barrier via M cells (Dramsi et al, 1996), although evidence for this was indirect and speculative. After crossing the intestinal barrier, *L. monocytogenes* locates mainly in the liver and spleen. In the liver, resident macrophages (kupffer cells) quickly kill a large percentage of the invading organisms, but those that survive enter and replicate mainly in hepatocytes (Gregory et al, 1992). *L. monocytogenes* can invade new cells through cell-cell spreading (see later) or by "normal" invasion after being set free, eg through listeria induced apoptosis of the hepatocyte or lysis of the hepatocyte by infiltrating neutrophils (Rogers et al, 1996). Subsequently, the bacteria are either cleared by the host, or undergo haematogenous dissemination to the brain (Berche, 1995).

### 1.6.1. Survival and Replication of *Listeria monocytogenes* in Host Cells

The intracellular life cycle of *L. monocytogenes* is one of the most studied of

recent years. Once internalised by a macrophage, or other cell type, *L. monocytogenes* must escape the vacuole it is contained by for two reasons: to avoid the killing mechanisms professional phagocytes employ, and to be able to multiply and spread to adjacent cells. For this purpose *L. monocytogenes* carries on its chromosome a gene cluster, the products of which are responsible for the virulent phenotype. The gene cluster contains, in the following order: *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*; the gene product of *prfA* being the positive regulator of the genes in the cluster, and also of some other virulence associated genes (see later). Figure 1.5 summarises the role the above mentioned genes play in pathogenesis, and for comprehensive reviews of the virulence gene cluster and its part in pathogenesis see Kuhn and Goebel (1995) and Goebel et al (1998).

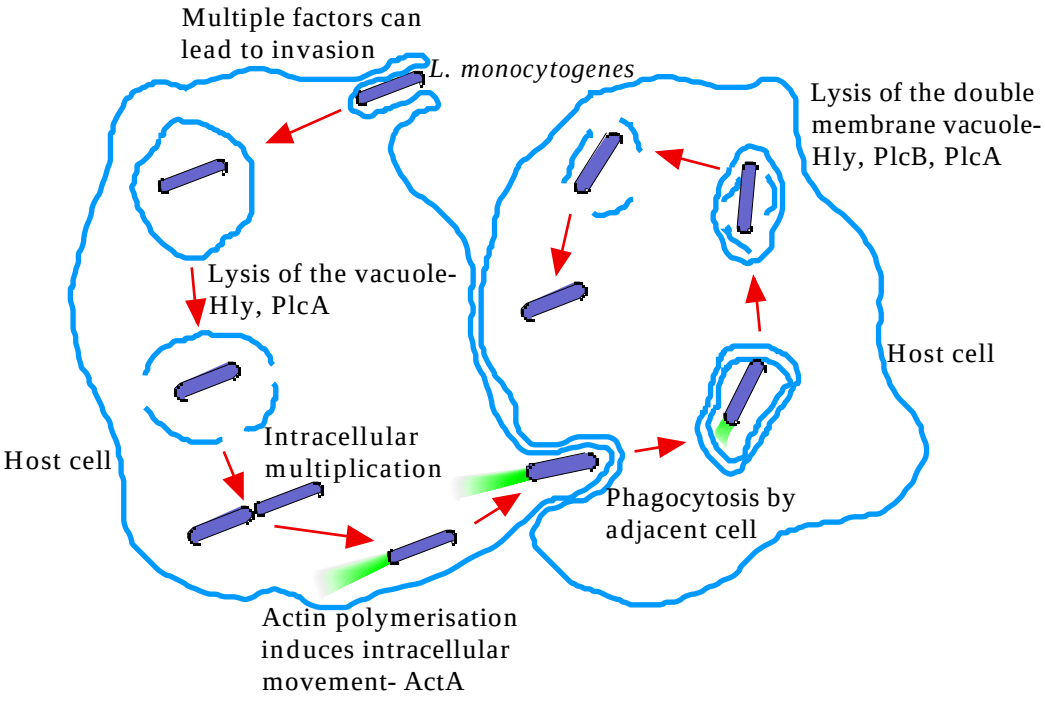


**Fig. 1.5.** Diagram showing the role of the virulence gene cluster of *L. monocytogenes*. The bacterium escapes from the host cell vacuole, propels itself into an adjacent cell creating a double-membrane vacuole and then escapes again to start the process once more. This was first described for *L. monocytogenes* infected macrophages, but is also true of other cell types.

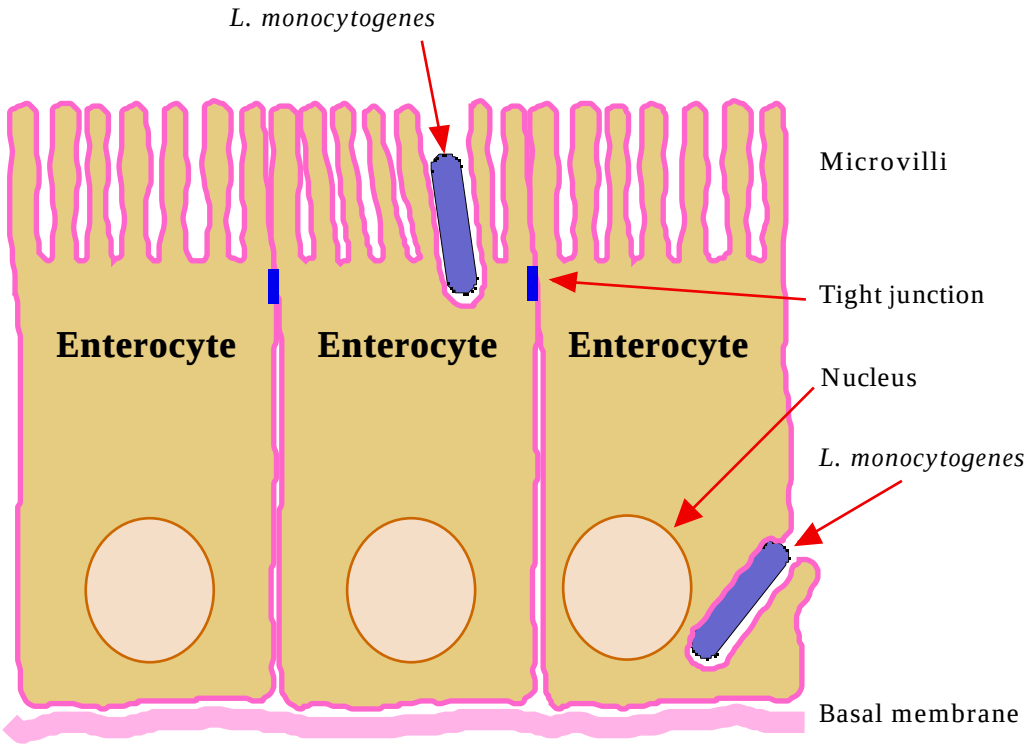


**Fig. 1.6.** Diagram showing the so called zipper mechanism entry of *L. monocytogenes* into non-phagocytic cells (enterocytes). Very little morphological change of the host cell accompanies *L. monocytogenes* entry, which is in stark contrast to *S. typhimurium* invasion. Entry has been reported from the apical side and the basolateral side of cultured epithelial cells.

### 1.6.2. Adhesins and Invasins of *Listeria monocytogenes*

*L. monocytogenes* is a pathogen that can direct its own internalisation into non-phagocytic cells, which has been termed a zipper mechanism of entry (see Fig. 1.6)- a phrase first coined for *Yersinia enterocolitica* entry (Isberg and Leong, 1990). Entry is in a morphologically less dramatic way than for *S. typhimurium*. Adhesion and invasion are often intimately associated as close proximity of the bacteria to the host cell is necessary to activate host cell signalling pathways which lead to uptake. To date, three bacterial factors seem to be involved in adhesion and/or invasion of non-phagocytic cells: the internalins, p60 and ActA.

#### 1.6.2.1. The Internalin Family of Proteins

The internalin gene (*inlA*) was discovered after transposon mutagenesis of *L. monocytogenes* and screening with Caco-2 invasivity assays (Gaillard et al, 1991). A transposon was found upstream of two open reading frames, now designated

*inlA* and *inlB*. When the *inlA* gene was cloned into the normally non-invasive *L. innocua* species, this organism exhibited an invasive phenotype. The genome of *L. monocytogenes* also contains other internalin genes, namely *inlC* (Engelbrecht et al, 1996); *inlC2*, *inlD*, *inlE*, and *inlF* (all four reported in Dramsi et al, 1997); and *inlG*, *inlH*, and *inlE* (all three reported in Raffelsbauer et al, 1998), and are often located in clusters, eg *inlAB*, *inlC2DE*, and *inlGHE*.

The predicted proteins from these genes exhibit a typical signal sequence and leucine-rich-repeat (LRR) sequences. LRR proteins are known to be involved in protein-protein interactions (Kobe and Deisenhofer, 1995), indicating an extremely broad range of possibilities for the internalins to interact with host cell proteins. Furthermore, antibodies targeted against the LRR region of InlA blocked entry of *L. monocytogenes* into cells expressing the InlA target, E-cadherin (Mengaud et al, 1996b). Except for InlC, the internalins have a membrane anchor portion (hydrophobic region) and a cell wall anchor both at the C-terminal end and are hence found mainly bound to the bacterial cell surface. Internalins are also found in *L. ivanovii* (Engelbrecht et al, 1998). As to the function of the internalins much is still under discussion, but the following has been documented in the literature.

**InlA** binds to human E-cadherin, a calcium dependent, cell-cell adhesion molecule that is found on the basolateral surface of epithelial cells (Mengaud et al, 1996a). Furthermore, when murine fibroblasts were transfected with L-CAM (the chicken homologue of E-cadherin), bacterial entry was seen to increase drastically. In addition, antibodies directed against L-CAM blocked bacterial entry of transfected fibroblasts (Mengaud et al, 1996a). Although the work described for the discovery of InlA and then its receptor, E-cadherin, is thoroughly and elegantly done, in the mouse model an *inlA* mutant unexpectedly shows only a slight difference in virulence after oral inoculation (Gaillard et al, 1991). This may be due to a non-recognisable E-cadherin homologue in murine cells. Furthermore, recent work has shown that many clinical isolates of *L. monocytogenes* have mutations in the *inlA* gene resulting in loss of the

membrane anchor in the protein (Jonquières et al, 1998), bringing into question the role of InlA in murine and clinical listeriosis.

**InlB** was first thought to mediate invasion of hepatocytes, but not enterocytes (Dramsi et al, 1995), but is now known to be at least in part responsible for invasion of numerous cell types, eg Vero cells (African Green Monkey kidney epithelial cell line), HEp-2 (human laryngeal epithelial cell line), HeLa (human cervical epithelial cell line), human primary endothelial cell cultures (Braun et al, 1998, Müller et al, 1998, Greiffenberg et al, 1998). Activation of the phosphoinositide 3-kinase p 85 alpha-p110 is among the early signalling events known to occur upon entry into the host cell (Ireton et al, 1996). This activation requires among other things, InlB (Ireton et al, 1996). However, the host cell receptor for InlB is not known. InlB has a novel cell wall anchor which is not found in the other internalins and hence InlB is found mainly bound to the bacterial cell surface (Braun et al, 1997).

**InlC** is a small, secreted, and strictly PrfA dependent internalin (Engelbrecht et al, 1996) unlike the *inlAB* operon which is only partially regulated by PrfA (Lingnau et al, 1996). A Δ*inlC* strain shows a significant decrease in virulence when tested in a mouse infection model, but very little difference in macrophage and epithelial cell invasion / survival assays (Engelbrecht et al, 1996). Together with its PrfA dependency, ie *inlC* is transcribed when the bacteria are in the vacuole / cytoplasm of the host cell, these data indicate no probable role in the invasion process. The biological function is unknown, but certain aspects are described later in this study.

**InlC2DE** and **InlF** were described by Dramsi et al (1997) with the genes being located in a cluster (*inlC2DE*) and alone (*inlF*). It seems that *inlE* and *inlD* are at least partially regulated by PrfA (Dramsi et al, 1997) indicating a potential role in the intracellular environment. Intriguingly, no significant differences were found in cell invasion / survival assays and in virulence in iv infected mice (Dramsi et al, 1997). This, however does not exclude a possible role in murine gut invasion or other cell tropism, or even animal tropism.

**InlGHE** are related to the aforementioned InlC2DE proteins in that it seems the genes encoding InlC2D have undergone homologous recombination to produce the *inlH* gene (Raffelsbauer et al, 1998). The *inlE* gene in both clusters is almost identical, and *inlG* seems to be a new internalin. Both clusters are located in similar positions on the *L. monocytogenes* chromosome. However, the genetic differences seen are due to strain variation. The data of Raffelsbauer et al (1998) also indicate that the *inlGHE* gene cluster is probably transcribed from a major PrfA independent promoter located upstream of *inlG.* A mutant which has lost the *inlGHE* cluster by an in frame deletion exhibits a significant loss in virulence in orogastrically infected mice, but not in iv infected mice when compared to the wild type strain, indicating a role in intestinal epithelial cell invasion (Raffelsbauer et al, 1998).

#### 1.6.2.2. The p60 Protein

p60 is a major secreted protein of *L. monocytogenes* having a molecular weight of 60 kD, and has been described as an invasion associated protein (Kuhn and Goebel, 1989). Spontaneously occurring rough mutants show reduced expression of p60 and form long filamentous chains in culture. Addition of purified p60 to these cultures restores normal morphology. p60 has murein hydrolase activity, and is probably involved in a late step of cell division (Wuenscher et al, 1993). Furthermore, sonicated rough mutants are defective for entry into certain cell lines, but addition of purified p60 restores invasiveness (Kuhn and Goebel, 1989). These results indicate a dual role for p60, namely an essential protein required for cell division, and an invasin.

#### 1.6.2.3. ActA

It has recently been shown that ActA, a listerial surface protein primarily involved in actin based intracellular motility (see Fig. 1.5), can bind to certain heparan sulfate proteoglycans (HSPGs) (Alvarez-Dominguez et al, 1997). HSPGs are found ubiquitously in plasma membranes of mammalian cells and have already been implicated as host cell receptors for microbial pathogens (reviewed

in Rostand and Esko, 1997). Alvarez-Dominguez et al (1997) have shown that binding and uptake could be significantly reduced by adding excess heparin or haparan sulphate (competitive inhibition), indicating *L. monocytogenes'* ability to bind heparan sulphate on the host cell surface. It was also shown that the predicted amino acid sequence and structure of the ActA protein contains clusters of different lengths of positively charged residues, which could mediate the establishment of ionic interactions with the sulphate groups of HSPGs. This was further confirmed by using an *L. monocytogenes* strain with an inactivated *actA* gene resulting in reduced binding and uptake. This observation that *L. monocytogenes* can bind to a ubiquitously found molecule expressed on the host cell surface may go some way in explaining the ability of *L. monocytogenes* to invade so many different cell types.

### 1.6.3. Entry of *Listeria monocytogenes* into Professional Phagocytes

For many species of bacteria internalisation into professional phagocytes would result in the bacteria being quickly killed. It is therefore particularly surprising to find certain bacteria, including *L. monocytogenes*, promoting their own uptake! *L. monocytogenes* can bind various components of complement, namely C1q (Alvarez-Dominguez et al, 1993) and C3b (Drevets and Campbell, 1991), and the resulting bacteria cell surface complex then binds the appropriate complement receptor on the macrophage membrane surface inducing uptake. InlA can also effect intimate binding of *L. monocytogenes* to the macrophage cell surface inducing uptake (Sawyer et al, 1996). As previously mentioned, ActA can bind heparan sulphate which is a ubiquitous molecule of eukaryotic plasma membranes (Alvarez-Dominguez et al, 1997). This molecule is, unsurprisingly, present on macrophages and was shown to play a significant role in invasion of a macrophage like cell line (Alvarez-Dominguez et al, 1997). Finally, lipoteichoic acids, which are components of the Gram positive cell wall, can bind to the type-1 macrophage scavenger receptor (Dunne et al, 1994) which may induce uptake of *L. monocytogenes.*

## 1.7. The Aims of this Work

The aims of this work were three fold:

1. To determine the effect the gene *slyA* has on virulence of *S. typhimurium*, particularly the colonisation of, attachment to, invasion of and post invasion effect on gut epithelial cells. Moreover, to identify genes possibly regulated by SlyA.

2. To establish the route of entry of *L. monocytogenes* into gut epithelia in terms of cell specificity and any morphological changes that are cocommitant.

3. To determine the biological function of *inlC* of *L. monocytogenes* in murine listeriosis