4. DISCUSSION

4.1. The Role of SlyA of *Salmonella typhimurium* in Virulence

Carter and Collins (1974) identified Peyer's patches as the primary route of infection by *Salmonella* in the mouse model, and more recent work by Jones et al (1994) and Clark et al (1994) have shown that M cells of Peyer's patches are the primary target cells for invasion by *S. typhimurium*. M cells may be identified as cells found exclusively in the FAE with shorter microvilli than enterocytes, a higher number of pinocytic vesicles and a flexible cytoskeleton which is often observed as lymphoid cells forming a basolateral pocket in the M cell (Smith and Peacock, 1980). M cells specialise in the uptake of particles from the lumen of the gut, and by a process of transcytosis, pass these particles to the underlying lymphoid follicles of the Peyer's patch, for subsequent antigen processing and presentation (Neutra and Kraehenbuhl, 1992). Jones et al (1994) compared an invasive *S. typhimurium* strain with a non-invasive strain in an ileal loop model, and found that the invasive strain exclusively attached to, and invaded, M cells, inducing M cell ruffling (Francis et al, 1993). After internalisation invasive *S. typhimurium* were cytotoxic for M cells, and later in the infection process adjacent enterocytes were invaded via the apical and basolateral sides causing whole stretches of epithelia to detach from the basal membrane, revealing the porous basal lamina beneath (McClugage et al, 1986). The purpose of the current study was to determine the role of SlyA in colonisation, invasion, cytotoxicity and survival using an intestinal mouse model.

Libby et al. (1994) recently proposed that SlyA represents a novel haemolysin of *S. typhimurium* and other *Salmonella* serotypes. This presumption was mainly based on the observation that the introduction of multiple copies of *slyA* into *Escherichia coli* leads to a haemolytic phenotype. Recent data demonstrate, however, that SlyA represents a new regulatory protein which seems to affect expression of genes in *E. coli* (Ludwig et al, 1995, Oscarsson et al, 1996) and also in *Salmonella*. This is in line with the notion that SlyA shares homology with

several other regulatory proteins of various Gram negative bacteria (Dehoux and Cossart, 1995). The most extended homology (28% identity and 51% similarity) exists between SlyA and MprA, a negative regulator of Microcins B17 and C7 in *E. coli* (Castillo et al, 1990, Oscarsson et al, 1996). It has been suggested that MprA may be a histone like protein that binds to DNA thereby altering local topology and hence transcription (Castillo et al, 1991). It has also previously been suggested that DNA supercoiling in *S. typhimurium* may have a regulatory effect on genes associated with invasion (Galán and Curtiss III, 1990) and responses to changes in environmental conditions (Karem and Foster, 1993). Oscarsson et al (1996) have shown that SlyA can bind to specific DNA sequences, which further adds weight to the possibility that SlyA is a transcriptional regulator, but as yet there is no direct evidence that SlyA is involved in altering DNA topology.

The previous work by Libby et al (1994) showing attenuated virulence of the *slyA* mutant in mice and reduced survival in murine peritoneal macrophages was performed with a *slyA* insertion mutant which carried the insertion close to the 3'-terminus of *slyA*. A possible residual SlyA activity could, therefore, not be ruled out. Two different insertion mutants have been used in this study, one carrying the insertion at the same position as the mutant described by Libby et al (1994) and the other close to the 5' end of *slyA* resulting in the generation of a mutant *slyA* containing a large internal deletion of 96 codons (with a second, inert *slyA* copy existing that has its promoter and first 9 codons replaced by suicide vector DNA). Both these mutants showed no detectable SlyA protein by Western blot analysis confirming a successful inactivation of the *slyA* gene (Fig. 3.4). It is interesting to note that the polyclonal anti-SlyA antiserum did not detect the larger (SLYAI) or smaller (SLYAII) mutated SlyA proteins from either mutant, but this may be explained by different folding of the proteins resulting in either antigenically dissimilar molecules from the WT protein, or that the mutant proteins are more susceptible to degradation. Both mutants behaved similarly in the above described virulence tests, indicating again that SlyA is functionally inactivated in both mutants. A polar effect of the insertions can be ruled out since

the gene downstream of *slyA*, namely *slyB*, is transcribed in the direction opposite to *slyA* and there is a transcriptional termination signal between the two genes which has not been replaced by vector DNA (Ludwig et al, 1995). The fact that the two *slyA* mutants and the isogenic WT strain of *S. typhimurium* have identical growth rates in LB broth and MOPS-Glucose minimal medium and no SlyA was detected in the mutants by Western blot analysis (Fig. 3.4) argues strongly against the possibility of SlyA regulating house keeping genes, but this possibility cannot yet be totally excluded.

It also seems likely from the SlyA kinetic data (Fig. 3.5) that the conditions within the host cell that prompt SlyA production are in some way analogous to the stationary phase of growth under laboratory conditions. However, SlyA production is not dependent on the stationary phase sigma factor RpoS (Buchmeier et al, 1997). Additionally, the two dimensional gel data reported here (Fig. 3.6) reveal a host of different proteins both up and down regulated in a *slyA* negative background. All these spots are possible candidates for explaining the observed virulence defect in the *slyA* mutants. Amino-terminal sequencing (Lößner, 1998) of one of the spots down regulated in a *sly*A negative background (see Fig. 3.6) has revealed 100 % identity with the SodB protein from *S. typhimurium* (only partial sequence known: GenBank Accession No. U09502), and extremely close identity to the SodB protein from *Escherichia coli* (GenBank Accession No. J03511). The molecular weight and isoelectric point of the spot is 25 kDa and pH 6.3, respectively (see Fig. 3.6), exhibiting very close similarity with SodB from *E. coli*: 23 kDa and pH 6.3. The SodB protein from *E. coli* is an iron superoxide dismutase and hence would be a particularly interesting candidate which could be involved in surviving the oxidative burst in the host cell vacuole. However, it has also been shown that SodB from *E. coli* does not have a drastic effect on macrophage survival assays (Papp-Szabo et al, 1993), hence SlyA probably also regulates other genes involved in virulence. Furthermore, Buchmeier et al (1997) report that SlyA is required for surviving oxidative stress, in vivo and in vitro. A *slyA* mutant *S. typhimurium* strain was shown to be

significantly more susceptible than the WT to hydrogen peroxide and paraquat (a redox-cycling agent which increases levels of superoxide radical), but not to nitric oxide donors (Buchmeier et al, 1997).

The orogastric experiments reported here extend previous data by Libby et al (1994) which suggested that a *slyA* mutation impairs intracellular survival of *S. typhimurium* within macrophages. The data show that two different *slyA* insertion mutants of *S. typhimurium*, SLYAI and SLYAII, are not significantly affected, as compared to the WT strain, in their ability to colonise the lumen of the small intestine, but significantly altered in survival in the Peyer's patches, reflected by the decreased number of viable bacteria found within Peyer's patch tissue after 2 and 7 days (Fig. 3.7). The slightly higher numbers of WT *S. typhimurium* found in the lumen of the small intestine after 7 days may be due to heavily infected Peyer's patch tissue being sloughed off into the small intestine, and hence reseeding this area with high numbers of bacteria, or reseeding via bile from the gall bladder. Theoretically, there are two possible reasons for the low numbers of viable bacteria of the *slyA* mutants found within Peyer's patch tissue after orogastric infection: (i) *slyA* mutants could have reduced survival capability, or (ii) they could have reduced invasiveness. Hence ligated ileal loop invasion assays were performed to determine invasion efficiency, and show that SlyA is not needed for invasion to take place, as both SLYAI and SLYAII strains invaded as efficiently as the isogenic WT (Fig. 3.8). Using transmission and scanning electron microscopy it was determined that the two *slyA* mutants induced ruffling and invasion of M cells in a morphologically and quantitatively similar manner as the WT strain, indicating again that *slyA* is not involved in the invasion process, but these two mutants caused significantly less tissue destruction than the WT strain (Figs. 3.9, 3.10, 3.12, and Table 3.1).

Interestingly, complementation of a *S. typhimurium slyA* mutant by a multicopy plasmid carrying *slyA* has been reported to restore survival in murine peritoneal macrophages, but not virulence when tested in the mouse model (Libby et al, 1994). This has been investigated further by studying the effect of a *S.*

typhimurium strain carrying a multicopy plasmid containing the complete *slyA* structural gene, *S. typhimurium*/pAL102, which successfully over expresses SlyA (Fig. 3.4). The orogastric data presented here show that the SlyA over producing strain is similar to both *slyA* mutants and the isogenic WT strain in its ability to colonise the small intestine, but significantly lower numbers of *S. typhimurium*/pAL102 were found within Peyer's patch tissue when compared to the parental WT strain (Fig. 3.7). In the ileal loop invasion experiments strain *S. typhimurium*/pAL102 was also found in similar numbers to the WT in Peyer's patches after 90 min (Fig. 3.8). Transmission and scanning electron microscopy revealed, using *S. typhimurium*/pAL102, ruffling and invasion of M cells, but significantly less destruction of the FAE after 180 min (Figs. 3.11, 3.12) than observed with the WT strain (Fig. 3.9). Nevertheless, *S. typhimurium*/pAL102 was observed inducing whole stretches of dying FAE 180 min after ileal loop infection (Figs. 3.11, 3.12), and 7 days after orogastric infection there were some cells (probably M cells) of the FAE destroyed (Fig. 3.13). It may be that the slightly lower number of M cells present after 7 days could explain the lower numbers of bacteria cultured from Peyer's patches after this time point, but it is likely that this accounts for only a small proportion of the difference. Nevertheless, this demonstrates that the SlyA over producing *S. typhimurium* strain, although obviously less aggressive than the WT, can still induce some tissue destruction in mice, which leads to the conclusion that the amount of SlyA within each bacterium is important for virulence.

The data described here indicate that SlyA does not affect expression of genes involved in adherence to M cells (or enterocytes) such as the recently described *lpf* genes (Bäumler and Heffron, 1995, Bäumler et al, 1996), or invasion, i.e. the *inv* and *spa* genes (Galán and Curtiss III, 1989, Groisman and Ochman, 1993), but rather the expression of genes required to exert the characteristic pathology in M cells, and/or the expression of genes involved in survival after M cells have translocated the bacteria to the lymphoid follicle. A large number of genes necessary for survival of *S. typhimurium* within macrophages have already been

identified and probably some of these are also required for survival in Peyer's patch tissue. Many of these genes are distributed throughout the chromosome of *S. typhimurium*, but some seem to be clustered together (Bäumler et al, 1994) and may form a topological DNA domain which could be globally affected in transcription by SlyA. The observation that multiple copies of the *slyA* gene, which lead to an over production of SlyA protein, in *S. typhimurium* also cause a partial attenuation in virulence in the mouse suggests that the amount of SlyA has to be precisely balanced in order to provide an optimal expression of the putative controlled genes. This is in line with the global regulatory function of SlyA on some of the already identified or still unknown macrophage survival genes, and on as yet unknown genes that are involved in the cytotoxic effect of *S. typhimurium* on M cells.

4.1.1. Future Research

It is interesting to note that after numerous attempts an in frame deletion was not possible within the *slyA* gene (Daniels et al, 1996). This leads to two possible conclusions: that a *slyA* mutant is lethal, or that the local DNA topology around *slyA* makes a double cross over recombination event very unlikely. Given that the two insertion mutants used here (SLYAI and SLYAII) are morphologically similar and grow at the same rate as the isogenic WT *S. typhimurium*, the possibility that SlyA regulates crucial house keeping genes is improbable. However, an in frame deletion mutant would make the results of further research into SlyA more convincing.

Which genes SlyA regulates, and how, are very important questions that have only been partly answered in this work. As previously stated, it is very likely that SlyA regulates other genes involved in virulence which are not yet known. Furthermore, it is known that SlyA is up regulated in late logarithmic and stationary phase of cultures grown under laboratory conditions, and that SlyA is not RpoS dependent. However, the environmental stimulus(i) that switches on SlyA production, and the genes involved in regulating SlyA are not known.

4.2. Interaction of *Listeria monocytogenes* with the Murine Host

MacDonald and Carter (1980) first showed that after oral inoculation of mice, *Listeria monocytogenes* associated predominantly with Peyer's patches of the small intestine. Since then there has been some debate as to whether *L. monocytogenes* also preferentially attaches to and invades M cells, found overlying intestinal lymphoid follicles, especially given that other intestinal pathogens invade via M cells (Jones et al, 1994, Perdomo et al, 1994 & Autenrieth and Firsching, 1996). The work of Rácz et al (1972) showed, in a guinea pig listeriosis model, that *L. monocytogenes* could enter intestinal epithelial cells from the apical side, but these authors only examined villus epithelium. This indicates that, at least in guinea pigs, *L. monocytogenes* can invade enterocytes, however, it does not exclude the possibility of more efficient invasion of M cells.

It is shown in this study, that the common *L. monocytogenes* laboratory strain EGD can reproducibly reach deeper organs after orogastric infection of BALB/c mice (Fig. 3.14), in a similar manner to that described by MacDonald and Carter (1980) and other authors (Marco et al, 1998, Pron et al, 1998). The increased numbers of bacteria seen in Peyer's patches, when compared to similar sized pieces of non-Peyer's patch intestine, yields three possible conclusions: i) *L. monocytogenes* is better equipped to invade the epithelial cell types found overlying the Peyer's patches (which would further indicate a role for M cells); ii) the bacteria are better able to survive and or multiply within Peyer's patch tissue when compared with villus tissue; iii) both i) and ii) are true.

To investigate which of these postulates is most likely, Peyer's patches and villus tissue were analysed using scanning electron microscopy (SEM). After oral infection of mice, very few rod shaped bacteria could be seen attaching to or invading enterocytes or M cells. It is important to note that the intestinal tissue was extensively washed before processing, leaving only very firmly bound bacteria. Only segmented filamentous bacteria could routinely be observed

overlying dome areas of Peyer's patches (Fig. 3.2), which has been described previously (Klaasen et al, 1992).

To increase the chance of seeing attachment or invasion of the bacteria, ileal loop tests were performed with a high dose of 10^9 CFU *L. monocytogenes.* Analysis of the intestinal tissue with SEM and scanning confocal laser microscopy revealed significantly more bacteria attached to the apical side of the epithelium than after orogastric infection. The bacteria were attached to enterocytes and M cells (Figs. 3.16, 3.17, 3.19), showing no particular preference for M cells. Also, no bacteria were seen in the crypt areas. These observations echo those of Rácz et al (1972) and Pron et al (1998). Nevertheless, the findings are slightly at odds with the work of Marco et al (1998) who found no *L. monocytogenes* interaction with M cells. However, this group used transmission electron microscopy which does not easily allow the observation of rare events due to the very small thickness and width of the sections analysed.

Very occasionally, numerous bacteria were observed invading an M cell (Fig. 3.18), causing significant apical membrane rearrangement, with no such alterations seen in enterocytes. M cell membrane alteration induced by *L. monocytogenes* has also been reported by Jensen et al (1998), however, it is not clear if this is part of the *L. monocytogenes* invasion process or if the M cell is under "stress" from already internalised bacteria. Given that this event was seen only rarely and ≥2 h after infection it is probably not an invasion specific event, but rather stress related.

Another very interesting finding was that whenever a part of the intestinal epithelium was seen to be damaged, numerous bacteria were attached to the exposed basement membrane (Fig. 3.20). These areas of epithelial damage were seldom seen, raising the question of whether the damage was induced by *L. monocytogenes*, as described by Jensen et al (1998), or were present before inoculation occurred. Damage was also only seen within the FAE, but due to less mucus production in this area, the FAE is more vulnerable to mechanical damage, eg sloughing off of cells by undigested, large food particles. To date, it is

known that *L. monocytogenes* can bind heparan sulfate proteoglycans (Alvarez-Dominguez et al, 1997), but whether it can bind other extracellular matrix (ECM) proteins is not known, eg collagens, laminin or fibronectin. However, it is a common trait of many other pathogens (Westerlund and Korhonen, 1993, Rostand and Esko, 1997). This observation may help to explain the apparent random nature of human listeriosis in otherwise healthy individuals, as all humans, and animals, periodically experience slight mucosal damage which is normally quickly repaired (Allen et al, 1993). That is to say, if an individual ingests high numbers of *L. monocytogenes* whilst mucosal injury is present, then he may be more vulnerable to *L. monocytogenes* invasion.

The extremely brisk translocation (~30 min) of bacteria into the blood, and from thence, livers and spleens, was unexpected and sheds a whole new light onto the invasion process of *L. monocytogenes* (Fig. 3.15). At least two cells have to be crossed by the bacteria to reach the blood: an enterocyte and an endothelial cell of the blood capillary. The bacteria found in livers and spleens was only a very small percentage (≤0.0001%) of the bacteria inoculated into the loop and seen in this light the result is, perhaps, not so startling. The data also supports the findings of Pron et al (1998) where they reported that 15 min after injecting 10^9 bacteria into a rat ligated loop, between $10^{4.4}$ and $10^{5.5}$ bacteria could be found in the livers and spleens of these animals. However, the experiments reported herein include: a *Bacillus subtilis* control organism (non-invasive), which was not able to cross the intestinal barrier, demonstrating the results are not artifacts of the ileal loop procedure; the livers and spleens were incubated with gentamicin after removal, minimising the chance of contamination; and finally, blood was taken via heart puncture to determine the route the bacteria take. These three things were not performed by Pron et al (1998). It has also been commonly thought that *L. monocytogenes* invades macrophages and is transported around the body via lymph, reaching its target organs via parasitised cells (Dramsi et al, 1996). Although these results do not exclude this as a possibility, they demonstrate a quicker alternative route- directly via blood. This indicates that a healthy

individual needs to ingest large numbers of bacteria to induce an infection, which seems to be confirmed by epidemiological data gathered from human outbreaks of *L. monocytogenes* (McLauchlin, 1993, Dalton et al, 1997). The efficient translocation of *L. innocua* suggests that the genes required for translocation are present on both the chromosome of *L. monocytogenes* and *L. innocua.* This therefore, rules out the virulence gene cluster of *L. monocytogenes* and, in fact, all PrfA regulated genes. The *L. innocua* chromosome does however, carry the p60 gene (Wuenscher et al, 1993) and may even contain some internalin genes (Gaillard et al, 1991, Dramsi et al, 1997) yielding the possibility that p60 or an internalin may facilitate this rapid translocation.

The findings reported here that describe the interaction of *L. monocytogenes* with a human like M cell culture (Kernéis et al, 1997) are of particular importance regarding the question: "which cell types are invaded by *L. monocytogenes* in man?". The very similar adherence, and distribution, of *L. monocytogenes* to the "FAE" of the coculture model (Figs. 3.21, 3.22) and the murine FAE (Fig. 3.19) suggests *L. monocytogenes* either uses identical adherence and invasion mechanisms in the murine and human host, or that *L. monocytogenes* contains multiple gene sets that enable invasion into different hosts or cell types. Furthermore, these experimental findings indicate that the preference *L. monocytogenes* shows in a murine intestinal model of listeriosis is similar to that seen in man, going some way in validating the murine listeriosis model.

The observation that attachment and invasion of *L. monocytogenes* is similar for FAE and non-FAE, ie villus epithelium, taken together with the higher numbers of bacteria seen in Peyer's patch tissue after orogastric inoculation, indicate enhanced survival and or replication within Peyer's patches. This has also been reported in the rat model of listeriosis (Pron et al, 1998). The reasons why this is so are unknown, but it is known that particles ingested by M cells bypass endosome-lysosome fusion giving the bacteria a better chance of reaching the lymphoid follicle intact. It is also possible that lamina propria lymphocytes, which are present in the normal gut mucosal tissue, but not in Peyer's patches, are

responsible for efficient killing of invaded *L. monocytogenes*, and hence better survival in Peyer's patches.

The data as a whole show that *L. monocytogenes* can invade FAE and villus epithelium, and attach to the basement membrane of damaged epithelium. Furthermore, *L. monocytogenes* can invade the apical side of differentiated epithelial cells, giving weight to the report of Karunasagar et al (1994) where these authors saw *L. monocytogenes* invading the apical side of Caco-2 cells. The major epithelial cell invasin of *L. monocytogenes* has been reported to be internalin (InlA), a leucine rich repeat protein (Gaillard et al, 1991). The eukaryotic receptor for internalin is E-cadherin, found on the basolateral side of differentiated epithelial cells (Mengaud et al, 1996a). It is, therefore, important to note that bacteria were never seen in the crypt areas, nor attached to the basolateral side of epithelial cells bordering damaged areas. The crypt areas contain epithelial stem cells and less differentiated enterocytes which may express E-cadherin on their apical side. Hence, it seems likely that internalin plays only a minor, or secondary, role in the intestinal stage of infection and the possibility that the genes required for invasion of polarised and differentiated enterocytic cells have not yet been identified. In addition, oral infection of mice with an *inlA* mutant has shown only small differences in the bacterial loads of livers and spleens (Dramsi et al, 1995), further supporting the notion that *inlA* is not the major intestinal invasin of *L. monocytogenes*. The work of Pron at al (1998) also demonstrates that neither the *inlAB* locus, nor *actA* (see also Ref. Alvarez-Dominguez et al, 1997), plays an important role in invasion of the rat intestinal epithelium, and further demonstrates that *L. innocua* is just as efficient in invading intestinal tissue as *L. monocytogenes*, indicating the genes required for invasion are not part of the virulence gene cluster, nor are regulated by PrfA.

The data herein complements the few published works covering *L. monocytogenes* invasion of differentiated intestinal cells and provides compelling evidence that, in contrast to other pathogens that invade the intestinal epithelia (for review, see Neutra et al, 1996 and Siebers and Finlay,

1996), *L. monocytogenes* does not need M cells to do this. Furthermore, it is shown that *L. monocytogenes* and *L. innocua* can quickly reach the deeper organs after intestinal infection, indicating the genes required for this are contained on the chromosomes of both species. The evidence derived from the mouse model is further supported by the similar results obtained from infected human M cell like coculture experiments, which probably more closely represents the human FAE (Kernéis et al, 1997).

4.2.1. The Role of InlC of *Listeria monocytogenes* in Virulence

Engelbrecht et al (1996) recently reported a new, PrfA dependent internalin, InlC. In this study, they demonstrated that InlC plays an important role in virulence when tested in a mouse infection model. The LD_{50} was increased for an *inlC L. monocytogenes* mutant by 1.5 Logs after iv infection, when compared to the isogenic EGD WT strain (Engelbrecht et al, 1996). However, only small, but reproducible, differences could be observed in invasion of the Δ*inlC* and WT *L. monocytogenes* strains in Caco-2 (human epithelial cell line) and J774 (murine macrophage cell line) cells (Engelbrecht et al, 1996), and human brain microvascular endothelial cells (Greiffenberg et al, 1998). Engelbrecht et al (1996) speculated that InlC may play a role in intercellular spreading, and given the leucine rich repeat domain, InlC probably interacts with host cell proteins (Kobe and Deisenhofer, 1995).

The data presented here show that a Δ*inlC* mutant is less able to replicate and survive in the livers, and to a lesser extent the spleens, of mice infected via the iv route, when compared to WT *L. monocytogenes* (Fig. 3.24). Mice infected orally display a similar kinetic (Jürgen Hess, personal communication) to the iv infected mice. To put the values into perspective, a Δ*inlAB* double mutant displays a less dramatic kinetic after iv infection (Gaillard et al, 1996, Dramsi et al, 1997), and has a lower LD_{50} than a Δ*inlC* mutant (Dramsi et al, 1997), revealing the significance of the role of InlC in virulence.

Because of the significant reduction in CFU of Δ*inlC L. monocytogenes* in the

livers of mice (Fig. 3.24), histological analysis of livers and spleens was performed. The first, and perhaps least surprising, observation was that the abscess like lesions in livers were considerably smaller in the Δ*inlC* infected mice when compared to the WT strain (Fig. 3.25). This has also been noted for an *inlAB* transposon mutant (Gaillard et al, 1996), although the effect of this large transposon was not studied and may itself influence virulence. Smaller lesions could indicate a defect in primary invasion of hepatocytes, or defective spreading, or an inability to escape the vacuole.

However, the Δ*inlC* strain induced more lesions that the WT (Fig. 3.25), which is a new and unexpected finding with regard to *L. monocytogenes* virulence factors. An *inlAB* mutant, for example, induces smaller and fewer lesions than the isogenic WT *L. monocytogenes* strain, even when mice are infected with extremely high doses of bacteria (Gaillard et al, 1996). Furthermore, the WT *L. monocytogenes* infected mice induced, later in the infection, considerably less lymphocyte infiltration than the Δ*inlC* strain (Fig. 3.25). Taken together, these data indicate a role for InlC in modulating host cell functions to hide from immune cells. As to with what, and how, InlC interacts with host cell constituents is not yet known, and the following text contains only a brief, speculative discussion.

Götz et al (manuscript in preparation) has shown that only very few bacterial species are able to replicate in the host cell cytosol after microinjection, *L. monocytogenes* being one of these species. Furthermore, a Δ*inlC* mutant is attenuated in spreading and intracellular replication, when compared to the WT (W Goebel, unpublished results). This leads to the intriguing conclusion that InlC could play a significant role in suppressing the inhibiting product(s) contained in the cytosol.

It is known that *L. monocytogenes* induces apoptosis in infected hepatocytes (Rogers et al, 1996) and that the *hly* gene product is responsible for inducing apoptosis in dendritic cells (Guzman et al, 1996), hence it is tempting to speculate whether InlC delays apoptosis. It is also known that *L. monocytogenes* induced

apoptosis of hepatocytes stimulates neutrophil chemoattractant release (Rogers et al, 1996). Hence, blocking, or slowing down, apoptosis would give the bacteria more time to replicate and spread to adjacent cells before neutrophils are called to an infected hepatocyte. This would hence explain the more numerous lesions in livers of Δ*inlC* infected mice, as the bacteria would become "visible" to neutrophils quicker than the WT strain.

InlC may modulate cytokine production of the infected cell to hinder chemoattractant release, but although bacterial perturbation of cytokine networks is well recognised (Kuhn and Goebel, 1994, and for a review see Wilson et al, 1998), nothing in this area is known about InlC.

Whatever the precise role of InlC, it is clear that it hinders a quick and effective immune response of the infected host giving the bacteria a survival advantage.

4.2.2. Future Research

As with much research, the experimental results seem to demand the answers to more questions than were there before! So it is with the data presented here.

The observation that *L. monocytogenes* does not adhere to or invade villus epithelium better than FAE, but that after oral infection more bacteria reside in the Peyer's patches is not explained in this work. Whether lamina propria lymphocytes play an important role or not should be investigated.

The finding that *L. monocytogenes* can bind very efficiently to the basement membrane is a very interesting phenomenon-which needs to be further studied. Which types of ECM proteins are bound by *L. monocytogenes*, and the gene(s) coding for the bacterial receptor are not yet known.

It is tempting to speculate as to the way InlC interacts with host cell constituents, but data is, at present, lacking. Whether InlC modulates cytokine production, cytoplasmic growth, or apoptosis, needs to be explored, and at the very least these theories need to be disproved.

4.3. Why Study Bacterial Interaction with the Host?

The two main reasons why research into host-pathogen interactions are important are: i) basic research often leads to new treatments for communicable diseases; and ii) research in this area can lead to bacterial strains that could be used as live antigen carriers.

4.3.1. New Treatments

Once, antibiotics were all that were needed to treat bacterial diseases, however this era is fast coming to an end (Travis, 1994). Bacterial resistance to known antimicrobial agents is increasing faster than the development of new agents. Although small peptide antibiotics may help in the short term (Hancock, 1997), it seems likely that whatever is thrown at bacteria, bacteria will eventually evolve that are resistant. For this reason, therapies using totally different, unrelated strategies, are needed.

Identification, and hence blocking, the eukaryotic cellular receptors that bacteria use to adhere and invade cells is one such new treatment strategy. This makes particular sense as adhesion of microorganisms to host surfaces is often the first step in the development of a disease (Finlay and Falkow, 1997). For example, a myriad of carbohydrate structures exist on potential host cell surfaces, and bacteria can have lectin like structures that bind these carbohydrates, leading to intimate association (Sharon and Lis, 1995). The resulting treatment would be administration of specific sugars corresponding to the targeted carbohydrate which would compete for binding to the bacterial lectin like structure (Zopf et al, 1996). Another less specific, but perhaps very effective, way of hindering colonisation by a pathogen is to use an organism that is a normal part of the microbiota. *Lactobacillus acidophilus* can tightly adhere to human enterocytes, thereby providing a physical barrier against other organisms wanting to colonise the intestinal epithelium (Coconnier et al, 1993). It seems that *L. acidophilus* does not specifically compete for receptors, but due to its size, and number, covers the enterocytes so well that the receptors are no longer physically accessible to the

invading pathogen (Coconnier et al, 1993). Anti-adhesion therapy could be used prophylactically to prevent infection, but also to clear a current infection of mucosal surfaces, particularly the intestine. Hence, research that determines the receptors of pathogens is vital, and the first step to finding the receptors is to determine if a particular cell type, eg M cells or columnar epithelial cells, are targeted by the bacteria, as is the case for *S. typhimurium*, but not *L. monocytogenes*.

Another area of potential therapy comes from the study of cytokine production by infected cells. There are two sides to this coin: sometimes the infected cells can kill the internalised bacteria only after stimulation with particular cytokines (Jones TC, 1996), whereas in other situations, the production of certain cytokines promotes immune mediated tissue damage giving the bacteria a better chance to invade than when no, or different, cytokines are produced (Perdomo et al, 1994). Hence, therapeutic administration of cytokines could enhance the immune response against a pathogen; or the administration of antibodies that bind the cytokine, or the cellular cytokine receptor, can block a harmful immune response. Although this work has not studied cytokine production, it is nevertheless, an extremely important area of research.

4.3.2. Live Antigen Carriers

”Prevention is better than cure” is a saying that, translated into the world of medicine, is: vaccination is one of the best prevention strategies against communicable diseases. The possibility that attenuated bacteria could carry antigens of common or dangerous diseases and after oral administration could elicit an effective mucosal immune response has been the subject of much research over the last few years (Neutra et al, 1996). *Salmonella typhimurium* is an excellent candidate for a carrier to prime the mucosal associated lymphoid tissue (MALT), as it specifically targets M cells of the Peyer’s patches. The results presented herein regarding the effect of a mutation in *slyA* of *S. typhimurium* reveals a very promising attenuation, as the *slyA* mutant strains still target M

cells, but then are quickly killed. *Listeria monocytogenes*, on the other hand, does not specifically target M cells, albeit that it can enter M cells, and hence would not be as efficient at priming the MALT. However, it has been successfully shown that oral administration of a recombinant *L. monocytogenes* strain expressing a tumour antigen can both protect mice from tumour induction, and promote tumour regression of established tumours (Pan et al, 1996). The work of Pan et al (Pan et al, 1996) also demonstrates that their recombinant *L. monocytogenes* strain induces splenic $CD8^+$ and $CD4^+$ lymphocytes, revealing another exciting potential vaccine carrier strain to induce the systemic immune system.