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MOLECULAR BASIS OF PATHOGENICITY IN ENTERIC BACTERIA

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) We have focused upon the genetic and molecular basis by which pathogenic microorganisms penetrate eucaryotic cells. Our studies show that the pathogenic <u>Versinia</u> share at least two distinct chromosomal loci, <u>inv</u> , and <u>ail</u> , that play a role in their entry into eucaryotic cells. The presence of <u>ail</u> sequences is correlated to an extraordinary degree with the clinical significance of <u>Versinia enterocolitica</u> isolates.					
We have also initiated a study to examine the capacity of <u>Salmonella choleraesuis</u> to invade and transcytose through polarized epithelial monolayers of Madin-Darby Canine Kidney (MDCK) cells requires active bacterial RNA and protein biosynthesis and was blocked by low temperature. Apical <u>S. choleraesuis</u> infection resulted, in an increase in paracellular permeability but the MDCK intercellular contacts were not significantly disrupted. Mutations affecting the ability of <u>S. choleraesuis</u> to adhere to, enter, and subsequently replicate within MDCK cells have been isolated by transposon insertion mutagenesis. <u>Kennedy</u>					
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Introduction

This report reflects our final research efforts under a contract with the U.S. Army Medical Research and Development command (USAMRDC). Work in my laboratory has been more or less continually funded by a contract with USAMRDC since 1966 when I joined the Georgetown University Medical School faculty. At that time the principal thrust of our work was directed towards understanding the rapid development of antibiotic resistance in enteric bacteria important in human disease. The recognition that this resistance was largely due to the dissemination of extrachromosomal elements of bacteria, plasmids, was exciting twenty years ago; it is still important today.

In order to effectively study bacterial plasmids, it became necessary to devise better methods to isolate and characterize the deoxyribonucleic acid of bacterial plasmids and the chromosomal DNA of their bacterial hosts. These activities are seen in the publications and reports which came from my laboratory at the time. In retrospect it is gratifying to see that some of the first molecular epidemiology and enteric bacteria molecular taxonomy came from collaborative studies between my laboratory at Georgetown and workers at WRAIR like Don Brenner. Also, we continued joint efforts with Dr. Sam Formal and Dr. L.S. Baron on various aspects of both shigellosis and salmonellosis.

The Army continued to support my work when I moved to the University of Washington in 1972. The move brought about not only a striking change in geographical location, but a change in the direction and kind of work that we attempted. The first major change was the beginning of our focus of the contribution of bacterial plasmids to bacterial pathogenicity. The seminal studies of H. Williams Smith showing that small bowel adherence and enterotoxin biosynthesis was plasmid-mediated in animal pathogens led us to document that this was also the case for human diarrheal disease. We were then able to exploit our expertise learned from our study of plasmid-mediated antibiotic resistance (R-plasmids) to examine two other classes of plasmids, Ent and Kad, which encoded for enterotoxins and adherence respectively.

The year 1972 also marked the discovery of recombinant DNA techniques. With continued support from the Army, from 1972 - 1981, we used cloning methodologies to further our examination of the determinants of bacterial enterotoxigenicity, adherence and the mechanisms by which antibiotic resistance genes could be disseminated among human pathogenic microorganisms. During this time we published characterizations of the heat-labile (LT) and heat stable (ST) enterotoxins of E. coli as well demonstrating the role of DNA transposition in the transfer of antibiotic resistance among bacteria as diverse as E. coli and Neisseria

gonorrhoeae. We also were able to establish the utility of DNA hybridization to detect pathogenic bacteria in clinical material using cloned determinants of pathogenicity.

The year 1981 brought another change with our move from the University of Washington to Stanford University and, once again, there was a change in the focus of our research. We abandoned our research dealing with antimicrobial resistance completely. Moreover, while our previous studies had dealt with the microbial colonization of host surfaces and toxigenicity, we now began to devote our efforts to examine the entry of pathogenic bacteria into eucaryotic cells that are not professional phagocytes, like epithelial cells, fibroblasts and endothelial cells. The existing models of viral infection suggested experimental feasibility. Moreover, the work by Sam Formal and his colleagues at WRAIR had established the utility of this experimental approach for the study of shigellosis. The study of the mechanisms by which Shigellae and the enteroinvasive E. coli entered cultured cells was clearly very complex, however, so we turned to another model system in which we had some previous success.

We initiated studies to examine the molecular means by which the pathogenic Yersinia enter eucaryotic cells. As described in our last annual report, we were successful in identifying a single protein of about 103,000 daltons which we call invasin which, when present in E. coli K-12 mediates parasite-directed uptake of bacteria by eucaryotic cells. In the sections that follow, I summarize our work dealing with bacterial invasion and describe the work done up to the conclusion of our contract. In the Discussion, I provide an overview of all of the work performed under this contract since our move to Stanford in 1981.

Results

1. Further work on the identification of invasin. We have continued to analyze bacterial strains harboring the Yersinia pseudotuberculosis invasin locus, inv, in order to investigate the mechanism of host cell penetration [1]. We have now shown that both E. coli and Y. pseudotuberculosis strains harboring an inv mutations were defective for entry into cultured human epithelial cells. Furthermore, molecular clones containing inv DNA and little additional DNA, converted E. coli K-12 into a microorganism that was indistinguishable from the parental Yersinia strain with regard to eucaryotic cell entry. Data from in-vitro protein synthesis indicated that a 103,000 protein was synthesized from inv saturating the coding capacity of the locus. The nucleotide sequence of inv was completed and shows an open reading frame corresponding to a protein of similar size. A comparison of the deduced invasin protein sequence with the 6102 sequences currently found in established gene libraries failed to disclose any significant protein homology. Nonetheless it is

clear that this single protein is compartmentalized on the outer surface of bacterial cells provides them with the potential for a unique interaction with the eucaryotic cell surface.

In further studies [2], we have constructed a series of translational fusions between the *inv* locus and the *lacZ* gene. Each *lac*⁺ fusion strain expressed a hybrid protein containing invasin at its amino terminal end. Analysis of these gene fusions allowed determination of the direction of translation of the *inv* gene which was similar to that deduced from the nucleotide sequence. Our previous studies had documented that *Y. pseudotuberculosis* invasion was temperature regulated. We have now shown that that control of the *inv* gene expression is also temperature regulated. *PhoA* gene fusions to *inv*, when present in *Y. pseudotuberculosis* were expressed at lower levels when bacteria were grown at 37C rather than at 28C. In contrast, similar fusions were regulated in a temperature-independent fashion in *E. coli*, as was the *inv* gene, itself. These data suggest that *Y. pseudotuberculosis* has chromosomally encoded trans-acting functions that normally thermoregulate the expression of *inv*.

2. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Most human disease is caused by *Y. enterocolitica*. Consequently, we have gone on to clone the *inv* gene of this species as well [3]. Unexpectedly, we found that there was an additional invasin protein synthesized by this species. This gene, which we call *ail* for adhesin-invasion-locus, provides host bacterial cells with the capacity to enter certain classes of cells (HEp-2, HeLa) with low efficiency and others (CHO and Cultured *Drosophila*) with high efficiency. The product of the *ail* locus is a 14,500 dalton surface protein. The nucleotide sequence has been determined ; the deduced protein does not bear recognizable homology with any other known protein. It is of considerable interest that the *ail* locus is found uniquely in the human pathogenic species of *Yersinia*. In addition, the genetic sequences surrounding the *ail* locus, about 10 Kb in all, is found exclusively only in *Y. enterocolitica* isolated from human or animal disease and not in strains from the environment. We believe that this *ail* region plays a major role in *Yersinia* pathogenicity and in the regulation of other virulence-associated genes, like *inv*.

3. Identification of regions on a 230-kilobase plasmid from enteroinvasive *Escherichia coli* that are required for entry into HEp-2 cells. Over the past few years we have examined comparatively the ways by which *Salmonella*, *Yersinia* and *Shigella* enter eucaryotic cells [4,5]. As a consequence of these studies we have examined certain specific aspects of the invasion capabilities of these pathogens. Certain strains of *Escherichia coli* can cause an invasive diarrheal disease in humans which resembles shigellosis. These strains share with *Shigella* species

the ability to enter and replicate within colonic epithelial cells and the ability to bind Congo red dye in vitro when grown at 37C. Like the Shigellae, they contain a large plasmid essential for virulence. We have examined a 230 Kb plasmid from such an enteroinvasive E. coli [6].

The invasion plasmid was genetically marked with an antibiotic resistance transposon and mobilized into an E. coli K-12 background. This plasmid conferred upon E. coli K-12 the ability to enter and multiply within cultured human epithelial cells as well as the ability to bind Congo red. Expression of these phenotypes required growth at 37C. Transposon mutagenesis was used to identify regions on the 230 Kb plasmid required for virulence. All transposon insertions which resulted in the loss of the ability to enter epithelial cells, as well as the ability to bind Congo red, were mapped to a single 25-kb BamHI DNA fragment. Subclones from this 25-kb region were tested for their ability to complement invasion in non-invasive derivatives. A subclone containing about 8 kb of the left end of the 25 Kb BamHI fragment was capable of complementing non-invasive mutants with Tn5 insertions in this region and restored to these non-invasive mutants the ability to enter epithelial cells.

One practical side light of this work was our identification of a 2.5 kb HindIII fragment from within the 25 kb virulence region which could be employed as a specific probe for the identification of both enteroinvasive E. coli and Shigella species in clinical material [7].

4. Penetration of Salmonella through a polarized MDCK epithelial cell monolayer. During our studies of the comparative abilities of enteric bacteria to invade tissue, we initiated experiments to examine the interactions between the pathogenic bacteria, Salmonella choleraesuis and polarized epithelial monolayers of Madin-Darby canine kidney (MDCK) cells grown on membrane filters [8]. Association of bacteria with the MDCK cell apical surface was an active event requiring bacterial RNA and protein synthesis, and was blocked by low temperatures. Salmonella were internalized within a membrane bound vacuole and exhibited penetration through, but not between, MDCK cells. About 14 Salmonella per MDCK cell crossed the monolayer per hour to the basolateral surface, yet the monolayer remained viable and impermeable to E. coli. Apical S. choleraesuis infection resulted in an increase in paracellular permeability but the MDCK intercellular contacts were not significantly disrupted. S. choleraesuis added to the basolateral aspect of MDCK cells invaded inefficiently, and only small numbers of S. choleraesuis penetrated into the medium bathing the apical aspects of the epithelial cells.

Subsequently, surface protein mutants of the invasive *Salmonella* were generated using the transposon, Tnp_{phoA}. Some 625 alkaline phosphatase fusion mutants *PhoA*⁺ were screened for their capacity to pass through (transcytose) polarized MDCK monolayers grown on membrane filters. Forty-two mutants were unable to penetrate this barrier. None of these mutants could adhere to or efficiently invade the monolayers but none of these mutations were within the recognized type 1 pili genes or the MRHA adhesin genes of *Salmonella*. Thus far, the mutations have been divided into six classes. Class 1 mutants had altered LPS O-side chains while class 2 mutants had defects in their LPS core. Mutants belonging to classes 5 and 6 did not decrease the transepithelial electrical resistance of polarized MDCK cell monolayers, in contrast to the parental strains and the other mutants. Mutants belonging to class 1 were less virulent in mice, while class 2 and class 4 and 5 were totally avirulent for mice. Mutants from class 3 and 6 were as virulent for mice as *S. choleraesuis*. These results suggest that the ability to pass through epithelial barriers may be an important virulence characteristic of *Salmonella*. These data further indicate that in *Salmonella*, bacterial adherence, internalization and monolayer transcytosis are closely linked events. We also demonstrated that a mutant with a decreased rate of intracellular replication can pass through the monolayer at rates similar to wild type *S. choleraesuis*.

Discussion

Since the conclusion of our work at the University of Washington in 1981, our chief intellectual concern has been the mechanisms of microbial pathogenicity and the physiological and biochemical aspects of the interaction between a parasite and its host. Within the past seven years, the advances of microbial genetics and molecular biology have permitted us to begin to understand the mechanisms by which specific microorganisms cause specific diseases. The challenge for us has been to dissect these genes and their products important in pathogenicity from those common traits found alike in both pathogens and non-pathogens. At the same time, it was important to understand that pathogenicity is multifactorial and that we could only focus upon a limited aspect of the infection process. We have focused therefore upon a common route of infection which is central to the virulence of several enteric bacteria - entrance into epithelial cells.

The entry of a microorganism into a host cell is a remarkable event entailing the transport of intact living bacteria through one or more complex eucaryotic membrane barriers. Our studies show that the pathogenic *Yersinia* not only share a common plasmid that is essential for disease production but also that they share at least two distinct chromosomal loci, *inv* and *aia*, that play a role in their entry into eucaryotic

cells. We don't yet know, but we suspect, that inv, and especially ail may be essential for entry into animal cells and the establishment of infection. At least the presence of ail sequences is correlated to an extraordinary degree with the capability of Yersinia enterocolitica clinical isolates to invade cultured epithelial cells and cause clinical infection.

Many of us thought that bacteria most often bound to epithelial cells by means of pili (fimbriae). However, not all bacterial adhesins are fimbriae, nor do they necessarily appear as visible organelles on the microbial surface. The ail and the inv gene products can be classified as adhesins since they mediate adherence of Yersinia and other bacteria to the eucaryotic cell surface. Unlike other previously characterized bacterial adhesins, they also mediate entry into a variety of animal cells. None of the common adhesins, even those important in enteric disease like K88 and CFA-1, mediate entry although they do permit excellent adherence to the same variety of animal cells.

Thus, it is not the magnitude of the bacterial binding to the mammalian cell surface that is important in bacterial entry. Rather, it appears that it is the presence or absence of specific receptors on the mammalian cell surface recognized by bacterial adhesins like inv or ail that determines the susceptibility of the host to infection and the likelihood that a given microorganism will become internalized. We know little about these precise receptors. It is an important project for the future.

The actual entry of Yersinia and other enteric pathogens into epithelial cells can be blocked by microfilament disrupting agents like cytochalasin D. This is a characteristic of phagocytosis seen in professional phagocytic cells. However, this form of endocytosis actually occurs in most animal cells. An important distinction between pinocytosis and phagocytosis is that the former is a constitutive event while the latter is a highly regulated inducible event. Thus, the phagocytic vacuole in an epithelial cell is formed in direct response to a particle binding to the appropriate receptor on the eucaryotic surface. One assumes then that inv and ail evolved to interact with a different class of receptors than other bacterial adhesins. As we begin to understand more about the functional basis for this recognition, we may gain considerable insight into host susceptibility and why some pathogens prefer certain animal species. It will also be instructive to determine the surface and other changes that occur when a host cell mutates or otherwise gains resistance to Yersinia infection.

We are just beginning to be able to focus upon bacteria as they emerge from their sojourn within a host cell. We believe that internalized bacteria follow a directed traffic pattern. It

is possible that the route followed by internalized bacteria is dictated by the nature of the initial surface encounter. If so, inv and ail provide considerably more than simply entry and the precise nature of the eucaryotic receptor recognized by these bacterial products becomes even more important. Alternatively, other specific bacterial products might direct, organize or simply subvert normal eucaryotic intracellular traffic mechanisms. Understanding these phenomena should be of equal utility to those who study microbial pathogenicity as it is to those who study the fundamental biology of animal cells. Also, it seems likely that bacteria that spend time within an intracellular environment may be very different in a variety of ways from those grown in laboratory culture media. It will be important to develop means to examine the functional differences between bacteria that replicate in different conditions. This is a fundamental point that has often been asserted by medical bacteriologists like Professor H. Smith; however, it is only recently that we have learned the correct means to measure such differences experimentally. In future it should be possible to exploit genetic fusions similar to those described here to achieve this end.

We were fortunate to have chosen the Yersinia as our initial experimental focus to study the process of bacterial invasion. While the inv and ail invasins are regulated, they nonetheless suffice by themselves to give an "invasion phenotype" in a well-defined laboratory strain. In contrast to these single gene products, it takes a comparatively enormous amount of Shigella or enteroinvasive E. coli DNA to achieve a similar phenotype in the identical laboratory strain. These data most eloquently speak to the point that the means by which Shigella, a highly adapted human pathogen, achieves entry into host cells is considerably different from that utilized by the Yersinia, which prefer hosts other than humans. Moreover, while the Shigella replicate preferentially within epithelial cells, the Yersinia really prefer the mononuclear cells in the underlying lamina propria. One might have thought therefore that the Salmonella might have resembled the Yersinia in their molecular strategy for cell entry. Not so. The capacity of Salmonella to cause infection and disease, even from our beginning studies reported here, are clearly as complex as the Shigella. The comparative examination of the molecular mechanisms used by different bacteria should provide a better understanding into the evolution of bacterial pathogenicity. It can not help but provide new strategies to prevent infection and disease.

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