Molecular Characterization of Bacterial Virulence Factors and the Consequences for Vaccine Design

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The pathogenesis of bacterial infections involves a complex series of interactions between the pathogenic micro-organisms and the host. The outcome of an infection will depend on a combination of factors including the virulence of the bacterial pathogen, the immune status of the host and the innate resistance of the host. In recent years we have learned to apply molecular techniques to the study of bacterial virulence. Molecular approaches allow us to identify and characterize at a structural, biochemical and immunological level particular bacterial virulence factors. The information we gain from such studies can be used together with *in vivo* and *in vitro* models to investigate the immune response to individual bacterial antigens. In the future these studies may lead to the development of novel immunoprophylactic, chemotherapeutic and diagnostic reagents. In this lecture I will present a personalized review of recent molecular studies on bacterial pathogens with particular reference to the potential for vaccine development.

Molecular studies on bacterial virulence determinants

Arguably one of the most important early projects using genetic approaches to study bacterial virulence was the work of H. Williams Smith and colleagues on the enterotoxigenic *Escherichia coli* of animals (Smith & Linggood, 1971). The approaches taken by Smith have served as a model for many subsequent investigations by other workers. Smith used simple genetic crosses to transfer putative virulence determinants including those for haemolysins, enterotoxins and adhesion fimbriae between *E. coli* strains to create isogenic pairs of strains differing in one or other factor. Of great importance was the fact that he used the relevant target host species for his investigations such as pigs or cattle and did not substitute these with less relevant, but more convenient species. His approach was made easier because the genes under study, e.g. K88 or K99 adhesion antigens, the heat-stable (ST) and heat-labile (LT) enterotoxins or haemolysins, were naturally encoded on plasmids. Now we are able to readily transfer naturally chromosomally encoded genes between species using *in vitro* DNA manipulation supported by classical genetic techniques. Smith's work highlighted the important role of adhesion antigens in promoting colonization of the small intestine by pathogenic bacteria and the role of the enterotoxins in inducing diarrhoea (Smith & Linggood, 1971).

Probably the first person to apply molecular approaches and gene cloning to the study of bacterial virulence was Stanley Falkow, whose group began cloning the virulence factors characterized so effectively by Smith. Falkow's cloning work on *E. coli* LT and ST played an important role in the subsequent molecular characterization of these important toxins (So *et al.*, 1976, 1978). While in Falkow's laboratory I began a study on the molecular characterization of *E. coli* adhesion antigen determinants. K88 are the adhesion fimbriae most commonly associated with porcine enterotoxigenic *E. coli*. K88ac was cloned on an 11.5 kbp DNA fragment into the multicopy vector pBR322 (Shipley *et al.*, 1981). Transposon mutagenesis, sub-

cloning, complementation analysis and *E. coli* minicells (Dougan & Kehoe, 1983) were used to map and characterize the polypeptides expressed from the cloned determinant (Kehoe *et al.*, 1981, 1983*a*; Dougan *et al.*, 1983*a, b*; Winther *et al.*, 1985). The K88ac determinant was shown to be composed of several closely-linked cistrons encoding the K88 fimbrial structural polypeptide and several polypeptides required for assembly and function of the adhesion fimbriae (Kehoe *et al.*, 1981). The minimal DNA required for functional K88ac expression was around 6 kbp although the natural K88ac promoter was not encoded on the cloned DNA (Dougan *et al.*, 1983*a, b*). This 6 kbp DNA fragment, when returned to wild-type *E. coli* strains, contained sufficient information to allow a non-pathogenic *E. coli* strain to efficiently colonize the small bowel of experimentally infected piglets (Dougan *et al.*, 1986). A variety of adhesion antigen determinants from enterotoxigenic *E. coli*, e.g. K88ab (Mooi *et al.*, 1979), K99 (Van Embden *et al.*, 1980), F41 (Moseley *et al.*, 1986) and 987P (Morrissey & Dougan, 1986), have now been cloned and characterized and the genetic organization of these determinants is remarkably similar (Morrissey & Dougan, 1985; De Graaf, 1988). Indeed, there seems to be a general model organization of fimbrial antigen determinants (Normark *et al.*, 1988).

Adhesion fimbriae play a vital role in the colonization of the mucosal surfaces of the host by bacterial pathogens. Adhesion mediated by such fimbriae involves specific recognition and binding to receptor molecules present on the surface membrane of eukaryotic epithelial cells. This binding to receptor can be inhibited by the presence of specific anti-fimbrial antibody. Thus vaccines have been developed based on purified preparations of adhesion fimbriae (Morgan *et al.*, 1978; B. Nagy *et al.*, 1978; L. K. Nagy *et al.*, 1978). For example, K88-based vaccines have been used in veterinary medicine for a number of years. The identification of adhesins on important pathogens will be vitally important to the development of subunit or acellular vaccines against bacterial infections. Recent work on the adhesins of *Vibrio cholerae*, the cause of cholera (Mekalanos *et al.*, 1988), and *Bordetella pertussis*, the cause of whooping cough (Weiss & Hewlett, 1984), serves to illustrate this.

In addition to adhesion fimbriae, toxins play a vital role in the disease process. We have cloned and characterized several toxin determinants from bacterial pathogens including the α -toxin of *Staphylococcus aureus* (Kehoe *et al.*, 1983*b*; Fairweather *et al.*, 1983), and the phospholipase C of *Pseudomonas aeruginosa* (Coleman *et al.*, 1983) and *Clostridium perfringens* (Leslie *et al.*, 1989). Cloned toxin genes can yield vital information about toxin structure and function and can be used as a start point to create strains harbouring defined mutations in the toxin gene within the natural host (Weiss *et al.*, 1983; Weiss & Hewlett, 1984; Foster *et al.*, 1988; Black & Falkow, 1987) or they can serve as the basis for the construction of vaccine strains (Mekalanos *et al.*, 1983; Kaper *et al.*, 1984). Toxin genes can also be readily manipulated to direct the expression of toxoids rather than toxins. An early example of this was the construction of a plasmid that directed the expression of the *E. coli* LT-B toxoid rather than the active LT toxin (Dallas *et al.*, 1979; Klipstein & Engert, 1981). More recent work has been undertaken using pertussis toxin (Black *et al.*, 1987; Gross *et al.*, 1989). The work of Neil Fairweather on tetanus toxin serves as an example of the manipulation of a toxin gene to create potentially useful immunogenic toxoids. Tetanus is an extremely potent toxin produced by *Clostridium tetani*. The current human tetanus vaccine contains semi-purified preparations of tetanus toxoid, inactivated by treatment with formaldehyde. Tetanus toxin is synthesized as a 150000 dalton polypeptide which is normally nicked to generate two polypeptide chains that are linked by disulphide bridges in the active toxin. Papain digestion of the toxin generates a 50000 dalton polypeptide, known as C-fragment, which is derived from the carboxyl end of the toxin. This fragment has been shown to be non-toxic, highly immunogenic and able to protect experimental animals against the lethal effects of toxin. Fairweather and colleagues cloned portions of the tetanus toxin gene in *E. coli* K12 and derived the entire DNA sequence of the gene (Fairweather *et al.*, 1986*a, b*). Using *E. coli* expression vectors they were able to construct strains of *E. coli* which expressed portions of the carboxy terminus of the tetanus polypeptide containing portions of the fragment C. These polypeptides were shown to be non-toxic and highly immunogenic in mice. Indeed mice vaccinated with some of these preparations were protected against tetanus toxin challenge (Fairweather *et al.*, 1987). Similar recombinant tetanus polypeptides may

provide the basis of future tetanus vaccines. Such polypeptides might eventually be synthesized by chemical means.

Although toxoids can be important components of acellular vaccines they may in themselves be insufficient to induce protection against some bacterial pathogens. *Bordetella pertussis* is the causative agent of whooping cough in young children. The pathogen colonizes the upper respiratory tract by attaching to the ciliated epithelial cells which line the bronchial tubes. The current whooping cough vaccine used in the UK is composed of inactivated whole-cell preparations. There has been an intensive search for an acellular whooping cough vaccine. *B. pertussis* produces an array of surface and extracellular proteins which play a role in virulence. These include pertussis toxin, fimbrial haemagglutinin (FHA), adenylate cyclase, fimbriae and outer-membrane proteins. The expression of these extracellular products is under the control of a central regulatory system called the *vir* locus, which apparently acts as an environmental sensor that directly regulates gene expression (Weiss *et al.*, 1983; Weiss & Hewlett, 1984). Preparations of inactivated pertussis toxin and FHA have recently been tested as an acellular vaccine in Sweden with somewhat inconclusive results. Thus the search is continuing for other candidate antigens for inclusion in new acellular vaccine preparations.

We have been working on a *vir*-regulated surface-associated protein antigen of *B. pertussis* known as P.69. Pavel Novotny and co-workers originally identified an equivalent protein antigen P.68 on the surface of the related animal pathogen *B. bronchiseptica*. Preparations of this antigen and monoclonal antibodies against it were able to induce active and passive immunity respectively in experimental animals (Novotny *et al.*, 1985). P.69 of *B. pertussis* was shown independently to have similar protective properties and to be equivalent to agglutinin 3 of *B. pertussis* according to the Eldering typing scheme (Brennan *et al.*, 1988). Amino-terminal sequencing of the purified P.69 and peptide fragments derived from it allowed DNA oligonucleotide probes to be synthesized whose sequence corresponded to the amino acid sequences. These probes were used to clone the gene for P.69 from *B. pertussis* (Charles *et al.*, 1989). Sequences of the gene revealed an open reading frame which encoded a 93478 dalton polypeptide that was apparently processed at the carboxy-terminal end to generate P.69. Examination of the protein sequence revealed two repeated sequences, one rich in glycine and the other rich in proline. These regions may help divide the protein into functioned domains. P.69 and P.93 protein equivalents have now been synthesized at high levels in *E. coli* and other hosts and such proteins are candidates for inclusion in acellular pertussis vaccines.

In concluding this section it is clear that the molecular approach is a powerful way to characterize potential bacterial virulence determinants and it is possible to use the information obtained to aid the development of new subunit vaccines. We can expect this information to eventually help design new chemotherapeutic agents.

The rational attenuation of bacterial pathogens

We have already discussed one route to the creation of attenuated derivatives of bacterial pathogens. By using modern genetic approaches it is possible to identify key virulence determinants and construct otherwise identical strains which lack the determinant. This approach is well illustrated by the enterotoxin-deficient *V. cholerae* strains. H. Williams Smith and coworkers were able to create attenuated variants of enterotoxigenic *E. coli* by curing them of plasmids. It is clear that if an attenuated strain is to be considered for use as a live vaccine the mode of attenuation should be well defined to aid the quality control of the vaccine. What if a pathogen has few readily definable virulence factors? How might attenuation be readily achieved? One alternative approach is to identify key metabolic pathways whose function is essential for a pathogen to survive *in vivo*. Of course the importance of such pathways may differ between different pathogens. Let us use *Salmonella* as an example.

Next to *E. coli* the *Salmonella* chromosome is the most completely mapped. In spite of this fact very few so-called virulence genes have been identified, let alone mapped. Lipopolysaccharide (LPS) is known to play an important role *in vivo* and strains defective in LPS biosynthesis have been used as live vaccines (Wahdan *et al.*, 1982; Levine *et al.*, 1987). However, few other virulence genes have been identified although many *Salmonella* spp. harbour a virulence-

associated plasmid which is now being characterized in detail. In the early 1950s Bacon and Burrows isolated auxotrophic mutants of *S. typhi* which were attenuated in mice (Bacon *et al.*, 1950). The attenuated auxotrophs were purine or *p*-aminobenzoic acid (PABA) dependent. Thirty years later Hoiseth & Stocker (1981) used modern genetics to construct *aroA* mutants of mouse-virulent *S. typhimurium* strains. *aroA* is one of several genes encoding enzymes which form the pre-chorismate biosynthetic pathway, the only route by which aromatic compounds can be synthesized by bacteria. *aro* mutants require PABA, dihydroxybenzoic acid and the three aromatic amino acids for growth. The *aro* pathway is not present in mammalian cells. When administered to mice *aroA* mutants of several *Salmonella* spp. were found to be highly attenuated. Subsequently *aroA* mutants were shown to be highly effective oral vaccines in several species (Hoiseth & Stocker, 1981; Stocker *et al.*, 1983; Mukkur *et al.*, 1987; O'Callaghan *et al.*, 1988). *aroC* (Dougan *et al.*, 1988), *aroD* (Miller *et al.*, 1989) and strains of *Salmonella* carrying combinations of *aro* mutants behave similarly *in vivo*, and thus vaccine strains harbouring several different *aro* mutations can be constructed. When administered by the oral route to mice *S. typhimurium aro* mutants can establish a limited infection in the reticuloendothelial system. This infection is cleared after several weeks and the animals remained immune to reinfection (Killar & Eisenstein, 1985; Maskell *et al.*, 1986, 1987).

S. typhimurium strains harbouring purine mutations behaved differently (McFarland & Stocker, 1987; Nnalue & Stocker, 1987; O'Callaghan *et al.*, 1988). Strains harbouring mutations before the inosine monophosphate branch point of the purine pathway such as *purE* are much less attenuated than strains harbouring mutations which act after the branch point, e.g. *purA*. *purA* strains are highly attenuated and they are unable to efficiently colonize the reticuloendothelial system following oral infection of mice (O'Callaghan *et al.*, 1988). They are also ineffective oral vaccines. Strains harbouring combinations of *aroA* and *purA* mutations are even more attenuated than *purA* strains (O'Callaghan *et al.*, 1988). Thus different auxotrophic mutations can give rise to attenuated strains which show major differences in their *in vivo* behaviour. This is emphasized further if the strains are administered intravenously (i.v.) to mice. *aro* strains, after an initial drop of about 10-fold in viable organisms recoverable from liver and spleen in the first 24 h period, appear to multiply slowly, about a 10-fold increase over the following 7–14 d, and are then cleared over the following few weeks. *Salmonella*-susceptible mice. *aro* strains, after an initial drop of about 10-fold in viable organisms recoverable from liver immunity against virulent *S. typhimurium* challenge. *purA*-harbouring strains seem to be more susceptible to killing than *aro* strains in the first 24 h but once established the strains persist for several weeks, without any accumulation in numbers of viable organisms. Animals vaccinated with *purA* strains can show protection in the early weeks following vaccination but this immunity is not usually long-term and varies according to the *Salmonella* background strain harbouring the mutation. *aroA purA* double mutant strains are very susceptible to killing in the early hours after i.v. inoculation, as much as 99.9% of the inoculum being cleared. Again *aroA purA* strains can persist at low levels for long periods of time but the strains do not induce protection against virulent challenge.

In summary, *aroA* strains are highly effective live vaccines in susceptible animals when administered orally or i.v., whereas *purA* and *aroA purA* strains are poor vaccines. It is possible to measure key immunological differences in mice receiving *aroA* vaccines compared to mice receiving *purA* vaccines i.v. in the early days after vaccination. For example, mice receiving *aroA* vaccines can control a challenge at 14 d with virulent *Listeria monocytogenes* whereas mice vaccinated with *purA* strains cannot (D. O'Callaghan, unpublished results). Both types of vaccines stimulate the production of anti-*Salmonella* antibodies. Thus these attenuated variants may prove to be useful for analysing immunity to *Salmonella* vaccines. There are obviously clear and important differences in the *in vivo* and immunizing properties of these differently attenuated strains.

Besides using auxotrophic mutations, in what other ways can we indirectly attenuate virulent *Salmonella*? Curtiss and colleagues recently demonstrated that *Salmonella* harbouring *crp*, *cya*, or *crp cya* double mutations were attenuated and effective oral vaccines (Curtiss & Kelly, 1987). The adenylate cyclase system of bacteria is a key regulatory function directly affecting

expression of a variety of bacterial genes. A number of different interlinked multiple gene regulatory systems or regulons have now been identified in bacteria. Some of these have been associated with the regulation of virulence genes, acting through central regulatory genes which apparently are able to monitor the environment. Examples of such virulence-associated systems include the *vir* system of *B. pertussis*, the *tox-R* system of *V. cholerae* (Taylor *et al.*, 1987) and the accessory regulatory gene associated with extracellular protein production in *Staphylococcus aureus* (Foster *et al.*, 1988). These interlinked regulatory pathways may be potential targets for creating attenuated strains.

An alternative approach to using previously characterized mutations as a route to attenuation is to introduce random mutations into the genome of a virulent pathogenic bacterium and use screening techniques to identify attenuated variants. This approach is illustrated by the work of Weiss *et al.* (1983) using Tn5 in *B. pertussis* and the work of Fields *et al.* (1986) using Tn10 in *Salmonella typhimurium*. Weiss & Falkow (1984) selected strains with altered phenotypes prior to showing loss of virulence whereas Fields *et al.* used prescreening with an *in vitro* macrophage killing assay before testing *in vivo* for attenuation.

Most virulence-associated proteins are likely to be secreted across the cytoplasmic membrane either onto the cell-surface or into the environment. TnphoA is a transposon which can be used to directly select for transposon inserts in genes for secreted proteins (Manoil & Beckwith, 1985). The use of TnphoA in studying bacterial pathogens has been pioneered by Mekalanos and colleagues (Taylor *et al.*, 1987). TnphoA is a derivative of Tn5 which has part of the left inverted repeat sequence replaced by the coding region for bacterial alkaline phosphatase, lacking the natural signal sequence. The transposon is engineered in such a way that if TnphoA transposes into a site within the open reading frame a gene fusion can form between the amino-terminal region of the interrupted gene and the transposon-encoded alkaline phosphatase. Alkaline phosphatase is only active when secreted, hence active alkaline phosphatase can only be expressed if TnphoA transposes correctly into the gene for a secreted protein. A simple chromogenic substrate can be used to differentiate between strains producing active compared to inactive alkaline phosphatase. Thus by using a simple plate screening procedure it is possible to enrich for TnphoA insertions into genes for secreted proteins.

Finlay *et al.* (1988b) employed TnphoA mutagenesis of *Salmonella cholerae-suis* to generate mutants which were unable to pass through (transcytose) polarized epithelial monolayers of Madin Darby Canine Kidney (MDCK) cells grown on membrane filters. The mutants isolated were unable to invade this cell-line, a property which is associated with the parental strain (Finlay *et al.*, 1988a). The 42 mutants identified were subsequently divided into classes according to the particular phenotypes they displayed. A number of these classes were defective in LPS biosynthesis and were attenuated in their ability to kill mice by the oral route. Some classes were smooth and retained mouse virulence whereas others were smooth but had reduced virulence for the mouse. Thus the ability to invade this particular eukaryotic cell-line did not correlate absolutely with *in vivo* virulence in the mouse. However, it is clear from this work that *Salmonella* strains possess surface-associated structures which promote invasion and transcytosis of eukaryotic cells.

Irene Miller and Carlos Hormaeche in collaboration with our laboratory isolated a series of TnphoA mutants of *S. typhimurium* and screened them directly for their ability to kill mice following oral infection (unpublished work). Out of 96 mutants screened, 15 were attenuated and nine of these showed defects in LPS biosynthesis. The six remaining attenuated isolates were smooth. Southern blotting was used to show that the transposon inserts in these mutants mapped to at least five different sites on the *Salmonella* chromosome. Further, the strains differed in their abilities to establish limited infections in the reticuloendothelial system of mice, suggesting that they harboured mutations affecting different stages in pathogenesis. From these and other studies it is clear that TnphoA and similar reporter transposons will prove to be invaluable tools for studying bacterial virulence.

aro mutations are an effective means of attenuating virulent *Salmonella* strains. Can the same approach be applied to other bacteria? The *aroA* gene of *E. coli* encodes the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (Duncan & Coggin, 1986; Maskell *et al.*, 1988).

This enzyme is highly conserved in bacteria, lower eukaryotes (Charles *et al.*, 1986) and even plants (Mousdale & Coggins, 1986). We have cloned and sequenced the *aroA* gene from *Yersinia enterocolitica* (P. O'Gaora and others, unpublished) and from *B. pertussis* (Maskell *et al.*, 1988). The cloned genes were used to construct *Y. enterocolitica* and *B. pertussis aroA* mutants by *in vitro* mutagenesis in *E. coli* followed by allelic exchange in the original pathogens. Both *Y. enterocolitica* (F. Bowe and others, unpublished) and *B. pertussis* (M. Roberts and others, unpublished) *aroA* mutants were attenuated in mouse model systems. Thus it appears that *aro* mutations may be a simple means of attenuating a range of pathogenic micro-organisms. Such mutants might prove to be invaluable tools for studying infection and immunity.

Live bacteria as carriers of heterologous antigens

The attenuated strains described above are capable of establishing limited infections. Depending on the strain, or the mode of attenuation, a variety of immune responses can be stimulated in the host. Attenuated *Salmonella* strains are of particular interest since they are capable, following oral feeding of live organisms, of stimulating an array of immune responses in the host, including local secretory antibody production, humoral antibody production and a variety of cellular immune responses (Dogan *et al.*, 1987 *a, b*, 1988). *Salmonella aroA* strains can stimulate strong immunity to virulent *Salmonella* challenge whereas *purA* mutants stimulate only a local response. Since *Salmonella* is related to *E. coli*, foreign genes expressed in *E. coli* can be shuttled into *Salmonella* using a variety of techniques. For example, most plasmid origins of replication which function in *E. coli* will also function in *Salmonella*. DNA can be introduced into non-virulent *Salmonella* laboratory strains and then shuttled using bacteriophages such as P22 into smooth vaccine strains. A whole array of potentially important antigens from various pathogens have been expressed in *E. coli* (Winther & Dogan, 1984). Some of these have now been moved into *Salmonella* vaccine strains. They include antigens from bacteria (Black *et al.*, 1987; Clements & El-Morshidy, 1984; Maskell *et al.*, 1986, 1987), viruses (Tite *et al.*, 1988) and parasites (Sadoff *et al.*, 1988).

Salmonellae are able to enter and survive within macrophages and other related cells. This may be one of the reasons why live *Salmonella* vaccines are such potent immunogens. I will use a few simple examples of the immunogenicity of *Salmonella aroA* strains expressing selected heterologous antigens. An attractive goal might be to construct a live attenuated oral *Salmonella* strain which could induce immunity against several enteric infections. As mentioned previously, both enterotoxigenic *E. coli* and *V. cholerae* produce potent heat-labile enterotoxins which play vital roles in inducing diarrhoeal symptoms in their respective disease syndromes. Both toxins are antigenically and structurally related (Clements & Finkelstein, 1978; Dallas & Falkow, 1979, 1980). They are composed of an enzymically active A subunit and a non-toxic but highly immunogenic B subunit. As discussed above, recombinant plasmids have been constructed which direct the expression of LT-B or CT-B. LT-B expression plasmids can be readily introduced into *Salmonella aroA* vaccine strains which express LT-B in a normal manner (Clements *et al.*, 1986; Maskell *et al.*, 1986, 1987). Mice vaccinated with *S. typhimurium aroA* LT-B-producing strains produce an anti-LT-B secretory IgA response along with high levels of anti-LT-B serum antibodies. These antibodies will neutralize heat-labile toxin activity in tissue culture cell-lines (Clements *et al.*, 1986; Maskell *et al.*, 1986, 1987). Further mice vaccinated with these constructs are immune to subsequent challenge with virulent *Salmonella*. Antitoxin immunity in itself is probably not sufficient to protect against enterotoxigenic *E. coli* or *V. cholerae* even if a strong local immune response is induced, and additional factors may have to be included in effective vaccines. Nevertheless this type of work represents a step towards the development of new enteric multivalent vaccines.

LT-B is secreted by *E. coli* and *S. typhimurium*. Do antigens have to be secreted to the surface of *Salmonella* vaccines in order to stimulate an immune response in the host? The answer is apparently not. β -Galactosidase can serve as a model internal non-secreted antigen. *S. typhimurium* is normally a lactose-negative organism and does not produce β -galactosidase. The functional *E. coli* gene for β -galactosidase can be introduced into *S. typhimurium aroA* vaccine strains and β -galactosidase-producing *Salmonella* can be used to vaccinate mice. Mice

receiving these live vaccines mount a humoral antibody response to β -galactosidase and also develop a delayed-type hypersensitivity characteristic of a cellular immune response (Brown *et al.*, 1987). Thus predominantly internal *Salmonella*-associated antigens can be recognized following exposure to live *Salmonella aro* vaccines.

LT-B provides an example of carriage of a bacterial antigen. What about antigens which originate from non-bacterial sources? David O'Callaghan and John Tite at Wellcome have carried out work with the nucleoprotein of influenza virus. Unlike the haemagglutinin, the internal nucleoprotein is highly conserved in terms of sequence and immunogenicity between different isolates of influenza virus. The nucleoprotein is one of the dominant antigens in the cytotoxic T-cell response to influenza virus in mice. Purified nucleoprotein is capable of inducing protective immunity in mice against a lethal challenge with certain virulent influenza viruses (Wraith *et al.*, 1987). The nucleoprotein gene from A/NT/60/68 influenza virus has been expressed in *E. coli* as a fusion to *cro* using the inducible λP_R promoter (I. Jones and others, personal communication). The nucleoprotein gene was introduced into a *S. typhimurium aroA* vaccine strain and a variant was selected which expressed the nucleoprotein constitutively at a high level (Tite *et al.*, 1988). In fact this strain is an ideal source for purifying the antigen. Mice vaccinated orally with the *Salmonella*-nucleoprotein construct developed good titres of anti-nucleoprotein antibodies in their serum in the weeks following vaccination. Mice infected subcutaneously developed a cytotoxic T-cell response to the nucleoprotein. This cytotoxic T-cell response was class II MHC gene-product restricted. Further investigations are now ongoing to see if the mice can be protected against a lethal challenge with the virus.

Conclusions

In this review I have attempted to illustrate, using selected examples, how molecular biology has changed the approaches taken to study bacterial virulence factors. By analysing in detail the virulence determinants of individual pathogens we have been able, in many cases, to piece together the complex way in which highly evolved pathogens interact with their host. This detailed information will in the future aid the development of new therapies against infectious diseases and other human and veterinary illnesses. Bacterial pathogens interact in a very sophisticated manner with eukaryotic cells and by learning more about these processes we can discover new ways of studying the cell biology of higher eukaryotes. In the future, the study of bacterial pathogens will not be reserved for microbiologists but the work will become entangled with many other disciplines. Thus the area looks to have an exciting future.

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