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The molecular basis for the virulence of bacterial pathogens: implications for oral vaccine development

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Mechanisms of pathogenesis

The underlying mechanisms by which pathogens cause disease have fascinated scientists since microbiology began to take shape in the last century. Gradual progress was made following the pioneering work of Pasteur and colleagues until by the early 1960s many infectious diseases were under control in developed countries, although even today this is far from the case in many nations in the world. The implications of such advances were enormous for the well-being of human populations, yet even 25 years ago we had only a superficial understanding of pathogenicity. With the rapid advance of the science of molecular biology it has been possible to take a new look at old problems and gain a fuller understanding of biological processes. This has been particularly true for the application of molecular approaches to studying infections and their consequences for the host. Infectious agents are now known to be involved in more disease processes than simple infections. They can precipitate autoimmune responses, leading to allergy or other severe forms of tissue damage, and they can cause cancers. What is becoming clear is that there is often a common mechanism underlying apparently unrelated pathogenic processes and in many cases an infectious agent is responsible for triggering events. As a consequence, by studying the molecular basis of infection we may learn far more about diseases in general than previously realized.

Pathogens have evolved in close association with their hosts. Highly evolved organisms such as mammals possess sophisticated mechanisms for controlling the colonization of tissues by pathogens. Likewise, pathogens have evolved mechanisms for colonizing the host as efficiently as possible even in the presence of mammalian defences. It is thus not surprising that pathogens often mimic normal host processes and signalling mechanisms to aid their colonization (Finlay & Falkow, 1989; Bliska *et al.*, 1993; Relman *et al.*, 1990; Sandros & Tuomanen, 1993). Hence pathogens may be utilized as useful minia-

ture models for analysing normal host functions. As we gain a more detailed understanding of pathogenic mechanisms it becomes increasingly possible to consider rational approaches as an option to designing novel interventive therapies. Such therapies include immunoprophylaxis or the use of chemotherapeutic agents. It is thus a strong possibility that the gathering of basic information on infectious disease mechanisms might lead to novel therapies against many diseases (Dougan, 1989). In this Lecture I will attempt to illustrate how studies on pathogenic bacteria may lead to the development of practical treatments for important diseases. I will concentrate on approaches to the development of novel systems for the oral delivery of vaccines. I will not attempt to review the literature and apologize for any missing references.

The oral route to vaccine delivery

From the early days of vaccine development the majority of vaccines have been delivered to the vaccinee using injection. This is a reliable method of delivering set amounts of antigen directly to the immune system. However, the majority of infectious diseases are initiated by pathogens entering the body via exposed mucosal surfaces. Perhaps as a consequence the immune system, which has evolved to protect the body from infections, has elaborate mechanisms for controlling pathogens at mucosal surfaces. Indeed a whole arm of the immune system, often referred to as the mucosal immune system, is dedicated to this purpose (Bienenstock *et al.*, 1978; McGhee *et al.*, 1992). Pathogens interacting with mucosal cells can elicit local and sometimes systemic immune responses. Although there is evidence for compartmentalization of the mucosal and systemic responses it is also clear that there is cross-talk. Further, there is evidence for the existence of a common mucosal immune system, involving the trafficking of immune cells between different mucosal surfaces, although its role in protective

immunity to infectious diseases is not well understood (McGhee *et al.*, 1992). Mucosal immune responses, typified by the production of secretory IgA, may play an important role in protection against certain diseases such as cholera and shigellosis (Holmgren & Czerkinsky, 1992; Levine *et al.*, 1983, 1988). It is recognized that an efficient way of stimulating mucosal responses is to deliver antigens directly to mucosal surfaces; injected vaccines are regarded as being poor inducers of mucosal responses. In the future, we may learn how to activate mucosal immune responses efficiently using other routes.

Although it may be desirable to deliver antigens via mucosal surfaces a major hurdle is that the majority of antigens are poorly immunogenic when presented in this fashion. Extensive studies have shown that very few pure antigens can stimulate efficient local or systemic immune responses when delivered mucosally. Indeed, there is evidence that repeated administration of high levels of antigens to surfaces may induce systemic tolerance. Exactly why most antigens are poor mucosal antigens is by no means clear. Specialized immune tissues are found associated with mucosal surfaces. Phagocytic cells, known as M cells, which are found overlying foci of lymphoid tissues, are involved in sampling antigens at these surfaces. The involvement of normal enterocytes is less clearly defined. How and why these cells respond to antigen is understood only at a descriptive level. Certainly, it is not understood how the connection is made between mucosal presentation and the stimulation of systemic responses.

Antigens that stimulate immune responses when delivered to mucosal surfaces can be classified into live and non-living and are referred to as mucosal immunogens. A classic example of a live mucosal immunogen is the Sabin polio vaccine (Minor, 1992). There are very few examples of true, non-living mucosal immunogens although the best example is cholera toxin and related molecules.

Live mucosal immunogens

Individuals recovering from infections acquired through mucosal surfaces often display both a local secretory and a systemic immune response to the pathogen. Thus live organisms can behave as mucosal immunogens. The creation of attenuated variants of mucosal pathogens is a potential route to mucosal vaccine development. Although this is a highly desirable goal it has not proved an easy one to reach for many diseases, particularly bacterial pathogens. For a vaccine to be accepted there must be a balance between potential reactogenicity, caused by limited colonization of tissues, and immunogenicity. For many diseases it is not easy to predict the behaviour of attenuated organisms, especially in man. A further potential problem is the stability of an attenuated variant. In the past the use of genetically imprecise or undefined attenuating lesions has led to problems with reversion to virulence or instability between vaccine lots. In the modern era it is generally accepted that fully genetically defined attenuating lesions should be employed in building candidate vaccine strains, although this is still not always adhered to.

Perhaps the best characterized live oral vaccine, the Sabin polio vaccine clearly illustrates many of the advantages and disadvantages of using live vaccines (for a review see Minor, 1992). The disadvantages can often be overwhelming. For example, BCG vaccine was originally used as an oral vaccine until a major accident tilted the preference in favour of parenteral vaccination.

Live oral *Salmonella* vaccines

Early studies

It has proved extremely difficult to develop effective and acceptable oral vaccines against diarrhoeal diseases even though mucosal presentation of antigen is potentially an effective way of producing a highly efficacious vaccine. Major efforts have gone into the development of oral cholera, dysentery and *Salmonella* (particularly typhoid) vaccines and only recently have these efforts begun to bear fruit (Edelman & Levine, 1986; Levine & Hone, 1992). A discussion on the development of an oral typhoid vaccine illustrates the type of problem encountered. An oral vaccine based on attenuated *Salmonella typhi*, the cause of human typhoid, or other salmonellae offers several attractions. Individuals recovering from typhoid often show strong local and systemic responses to the bacteria. In addition, there is evidence for the presence of a strong cellular response. This potent immunogenicity has attracted attention to the use of attenuated salmonella as vectors for delivering heterologous antigens to the immune system (Curtiss *et al.*, 1989; Charles & Dougan, 1990; Chatfield *et al.*, 1990).

Early attempts to develop non-living oral vaccines against typhoid were short-lived as low efficacy was encountered. Instead, the preferred choice for vaccination became the parenteral route using inactivated whole bacterial preparations. This vaccine showed reasonable but variable efficacy and was (and still is) highly reactogenic. Reactogenicity has been improved by the development of a purer parenteral vaccine based on purified Vi antigen (Acharya *et al.*, 1987).

The modern search for a live oral typhoid vaccine perhaps began with the evaluation of streptomycin-dependent strains of *S. typhi* as oral vaccines. These strains grow poorly in the absence of the antibiotic (Reitman, 1967). Early encouraging results obtained with non-lyophilized vaccines were not repeatable when attempted with lyophilized preparations, a step essential for practical vaccine development. This early example illustrates the many pitfalls that can overtake vaccine developers. In the late 1950s the question of how to attenuate a highly pathogenic *S. typhi* strain was not a simple one as few virulence-associated traits had been identified. A further problem was the poor infectivity of *S. typhi* for hosts other than humans. For this reason many investigators in the field turned to the murine model of salmonellosis to seek clues. Mice can be infected with some strains of *Salmonella typhimurium* and other serotypes. These strains are highly mouse-virulent and rapidly kill genetically susceptible animals through an invasive infection, resembling typhoid

in many ways (Collins, 1974; Hormaeche, 1979). While studying this model Rene Germanier noticed that strains of *S. typhimurium* harbouring mutations defective in lipopolysaccharide biosynthesis were attenuated. In careful studies he showed that *S. typhimurium* harbouring *galE* mutations, defective in the enzyme UDP-galactose epimerase, were not only attenuated but were highly immunogenic (Germanier & Furer, 1971). Germanier decided to build upon this information and attempt to develop an oral typhoid vaccine based upon *S. typhi* attenuated by *galE*. At the time it was not possible to use modern molecular genetics to isolate mutants so instead Germanier selected his mutants using nitrosoguanidine treatment. After intensive selection studies, involving the isolation of *galE* variants of different sensitivity to galactose, he produced candidate vaccine strains (Germanier & Furer, 1975).

It is one thing to test a live typhoid vaccine in mice but quite another to assess safety and efficacy in humans. It requires a facility capable of challenging well-informed volunteers under close medical supervision and in a contained environment. Such studies are extremely expensive and even today only a few specialized centres can carry them out. Germanier carried out his studies in collaboration with Myrone Levine and others at the Center for Vaccine Development in Baltimore (Levine *et al.*, 1987a). Extensive studies in volunteers eventually established safety and efficacy and the vaccine strain, known as Ty21a was field-trialled in Egypt and South America (Waderman *et al.*, 1982). Work carried out over more than a decade showed that the vaccine had moderate efficacy if taken as three doses. Several modifications in vaccine formulation were required to establish optimal vaccine presentation (Levine *et al.*, 1987a). The vaccine is now licensed in many countries.

The search for a genetically defined vaccine

Although Ty21a is now sold as an oral typhoid vaccine the search has intensified for the development of a genetically defined live vaccine. This work has been stimulated by several factors including the potential use of *Salmonella* as a carrier. Ty21a has the disadvantage of being a multi-dose vaccine. It is also genetically undefined. Several other phenotypic differences other than those explained by *galE* exist between Ty21a and parental Ty2. The story was further complicated by the observation that at least one strain of *S. typhi* harbouring a defined *galE* mutation was still virulent in man (Hone *et al.*, 1987, 1988b).

Work by Bacon and Burrows in the 1950s had shown that certain auxotrophies could attenuate pathogens, including dependence on purines as well as other aromatic compounds including para-aminobenzoic acid (Bacon *et al.*, 1950). Although these observations remained dormant they were not forgotten and Bruce Stocker re-initiated studies using the murine typhoid model combined with modern genetics (Hoiseth & Stocker, 1981). Hoiseth and Stocker showed that *Salmonella* harbouring transposon-generated mutations in the gene *aroA* were highly

attenuated. This gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase vital for functioning of the chorismate pathway essential to bacteria for the biosynthesis of the aromatic ring (Pittard, 1990). Further, these strains were clearly excellent single dose oral vaccines. We were able to show that similar mutants of *S. typhi* were also attenuated (Dougan *et al.*, 1986). Stocker and colleagues went on to construct *aroA* mutants of *S. typhi* but decided for safety reasons to include *purA*, a second attenuating lesion. *S. typhi aroA purA* mutants were tested in Baltimore but although they were attenuated and non-reactogenic they were also poorly immunogenic (Levine *et al.*, 1987b). Experiments conducted in my laboratory at Wellcome by Duncan Maskell and David O'Callaghan went some way to explaining the observations found in humans (O'Callaghan *et al.*, 1988). They constructed a series of derivatives in mouse virulent *S. typhimurium* strains that harboured stable mutations in either *aroA*, *purA* or in both *aroA* and *purA*. Careful and detailed analysis of the persistence and immunogenicity of the otherwise isogenic strains showed that the *purA* lesion was more attenuating than *aroA* and that a combination of both *aroA* and *purA* further attenuated. Indeed *aroA purA* mutants had an unusually long, low level persistence in Balb/c mice but failed to induce immunity, probably because of their inability to induce critical anti-*Salmonella* cellular responses (O'Callaghan *et al.*, 1990). These experiments illustrated that important lessons could be learnt from the mouse model. We constructed *aroA purA* mutants of *S. typhi* and conducted a small volunteer study, in conjunction with Dr David Tyrell and the Common Cold Unit in Salisbury (our unpublished results). To my knowledge this is the only live *S. typhi* vaccine trial to have been conducted in the UK. We obtained very similar results in volunteers to those obtained by Levine and colleagues. At present there are no facilities in the UK where similar trials can be conducted.

The disappointing experiments with the *S. typhi aroA purA* vaccines in volunteers meant that other secure methods for attenuating *Salmonella* had to be identified. We, together with Carlos Hormaeche, showed that double *aro* mutants harbouring lesions in the *aroA*, *aroC* or *aroD* genes of the chorismate pathway were attenuated to a similar degree even if two or three mutations were combined in the same strain (Dougan *et al.*, 1988; I. Miller *et al.*, 1989a). These results encouraged a molecular characterization of the *aro* genes (Charles *et al.*, 1990a; Chatfield *et al.*, 1990; Servos *et al.*, 1991) of *Salmonella* in order to construct defined candidate vaccine strains where the full extent of deletions within genes was known and no foreign DNA was introduced into the strains. Initially, double *aro* mutant strains were evaluated in cattle and shown to be excellent single dose oral vaccines against salmonellosis even in cattle as young as 2 weeks old (Jones *et al.*, 1990). Strains of *S. typhi* harbouring two *aro* mutations were constructed in collaboration with Myron Levine and he went on to test the vaccines in volunteers with extremely encouraging early results (Hone *et al.*, 1991; Chatfield *et al.*, 1992a). These strains and derivatives are currently undergoing extensive characterization at

the Center for Vaccine Development in volunteers for immunogenicity and reactogenicity (Tacket *et al.*, 1992a).

In the past few years extensive analysis of the *Salmonella* genome has identified an array of genes required for expression of the virulence phenotype (Fields *et al.*, 1986; Finlay *et al.*, 1988; Finlay & Falkow, 1989; I. Miller *et al.*, 1989b). Thus there are a bewildering number of genes or combination of genes that might be considered for use as attenuating lesions. Very few examples exist where defined lesions have been evaluated either alone or in combination in terms of potential use in vaccine construction. Perhaps the best characterized outside of *aro* vaccines are the *cya crp* strains isolated by Curtiss and colleagues which have now also been tested in volunteers (Curtiss & Kelley, 1987; Tacket *et al.*, 1992b).

Other lesions that have been tested in model systems, include *phoP* (S. Miller *et al.*, 1989), *ompR* (Dorman *et al.*, 1989; Chatfield *et al.*, 1991) *htrA* (Johnson *et al.*, 1991; Chatfield *et al.*, 1992b; Strahan *et al.*, 1992) and *bemA* (Benjamin *et al.*, 1991). The major bottle-neck for testing such vaccines is clearly the availability of suitable facilities for administering live recombinant bacteria to volunteers. Potentially the major practical block to producing an acceptable genetically defined live typhoid vaccine will be obtaining the balance between good immunogenicity, where you have production of cytokines and other potent mediators, and reactogenicity which may be associated with a strong immune response. Information from volunteers is the only way to obtain answers.

The detailed genetic investigations carried out on attenuating lesions in *Salmonella* are proving to be a strong stimulus for work in other bacterial pathogens. For example, the *aroA* gene has now been isolated from a variety of bacteria and in some instances mutants have been shown to be attenuated (Roberts *et al.*, 1989; Bowe *et al.*, 1989; O'Goara *et al.*, 1989; Chamberlain *et al.*, 1993; Karnell *et al.*, 1993).

Salmonella strains as carriers

Since *Salmonella* strains are highly immunogenic when delivered orally their potential as carriers for delivering heterologous antigens to the immune system has been extensively investigated. Early work carried out using Ty21a and Shigella antigens was encouraging but also disappointing, highlighting the problems of using the poorly genetically defined strains (Black *et al.*, 1987). To date, an ever increasing number of antigens have been expressed in *Salmonella* vaccine strains and characterized to varying degrees (Curtiss *et al.*, 1989; Poirier *et al.*, 1988; Sadoff *et al.*, 1988; Tite *et al.*, 1988, 1990; Schodel *et al.*, 1990). *Salmonella* are closely related to *Escherichia coli* and similar genetic manipulation systems can be utilized in both organisms. One of the earliest antigens to be delivered by *Salmonella aro* mutants was the B subunit of the heat-labile enterotoxin (LT-B) of *E. coli* (Clements *et al.*, 1986; Maskell *et al.*, 1987). Using this antigen it was possible to detect both systemic and secretory immune responses to the antigen. LT-B is a secreted protein but

it was quickly shown in Carlos Hormaeche's group at Cambridge that the immune system could also recognize, in terms of both humoral and cellular responses, antigens expressed in the *Salmonella* cytoplasm (Brown *et al.*, 1986). This observation came as a surprise to many people who believed antigens would have to be expressed at the bacterial cell surface in order to be recognized. The early experiments with LT-B uncovered some of the problems with the system that would be regularly encountered later on. Duncan Maskell initially expressed the LT-B gene from a plasmid using the *tac* promoter but found that the plasmid was rapidly lost *in vivo* as the *Salmonella* vaccine colonized the mouse reticulo-endothelial system (Maskell *et al.*, 1987). In order to utilize the *tac* promoter it was not possible to regulate expression using the *lac* repressor as bacteria persisting in the host could not be induced using gratuitous inducers. The presence of the *lac* repressor drove expression down to such an extent that although the plasmid was stabilized no response against the heterologous protein was detected (Fairweather *et al.*, 1990). Thus the use of expression systems designed for routine laboratory use such as *tac* (*lac* induction) or λ p_L (heat induction) are flawed in terms of their value for live vaccine development.

Clearly, any form of instability is unacceptable if large vaccine lots are to be manufactured. Very few of the many papers describing the use of *Salmonella* as a carrier address this issue. In addition to instability the presence of antibiotic resistance determinants or blocks of uncharacterized foreign DNA would create problems for legislators. While constructing our candidate *S. typhi* vaccine strains we went to great lengths to eliminate foreign DNA or drug-resistance markers from the final candidate vaccine strains (Chatfield *et al.*, 1992a). If *Salmonella*, or indeed any other live vector, is to be used as a practical vaccine these issues must be addressed. Groups have begun to address the issue of plasmid instability. One approach is to move genes onto the bacterial chromosome (Hone *et al.*, 1988a; Strugnell *et al.*, 1990). Obviously, because the genes are single copy, expression levels are driven down but it is still possible to obtain immune responses which can be protective in model systems, against the heterologous protein, for example the pertactin protein of *Bordetella pertussis* (Charles *et al.*, 1988, 1990b; Strugnell *et al.*, 1992). Nakayama *et al.* (1988) developed an elegant genetic method, based on the presence of a gene required for bacterial host survival. They constructed an attenuated *Salmonella* carrier harbouring a lesion in the *asd* gene. Lack of diaminopimelic acid in the medium is lethal to *S. typhimurium asd* mutants. In Curtiss's system the *asd* gene is placed on the plasmid harbouring the heterologous gene. Thus loss of the plasmid results in removal of the plasmidless progeny from the population.

We utilized a different approach to solve the problem. The earlier work with LT-B had shown that plasmid stability *in vivo* was greatly influenced by the type of promoter utilized to express the foreign gene. Ideally, it would be desirable to use a gene that turned on efficiently only when the bacteria entered the host or even when they interacted

with immune cells. Some bacterial promoters are known to be sensitive to changes in the extracellular environment around the bacterial cell (Charles & Dougan, 1990). For example, *ompR*-regulated promoters are sensitive to changes in osmolarity. We had been trying for some time to develop a single dose oral tetanus vaccine based on *Salmonella*. Neil Fairweather in our team at Wellcome had cloned and sequenced the tetanus gene and he and others had obtained high level expression of Fragment C, a non-toxic but highly immunogenic domain of the toxin, in several systems (Fairweather *et al.*, 1987). Recently, he and Ian Charles had utilized the *nirB* promoter to obtain expression of Fragment C regulated by levels of oxygen in the medium (Oxer *et al.*, 1991), following a conversation with Professor Cole from Birmingham University (Peakman *et al.*, 1990). Steven Chatfield in our group decided to monitor the behaviour of an *S. typhimurium* vaccine strain harbouring a plasmid driving expression of Fragment C from *nirB*. Earlier attempts to develop a single dose oral tetanus vaccine based on *tac* expression systems had met with partial success (Fairweather *et al.*, 1990). Unlike the experience with the *tac* promoter oral feeding of the hybrid vaccine strain to Balb/c mice resulted in almost perfect stability of the plasmid in bacteria recovered from the mice, even many days after inoculation. Mice routinely efficiently sero-converted to Fragment C and were solidly protected against tetanus challenge (Chatfield *et al.*, 1992c). These studies were extended to construct candidate *S. typhi* vaccine strains expressing Fragment C from the chromosome (Chatfield *et al.*, 1992a).

The *nirB* system has now been further exploited with many antigens being stably expressed from this promoter in *Salmonella* vaccine strains. Experiments with other *in vivo* inducible promoters have also been extremely encouraging (S. Chatfield, M. Roberts & Li Jing Li, unpublished results).

Non-living oral vaccines

Although live vaccines might be the quickest way to achieving practical goals the long-term aim has to be their replacement by non-living systems since arguably a perfect oral vaccine would have to be non-living and chemically defined. This is particularly true in developing countries where lack of an efficient medical infrastructure highlights the danger of HIV transmission and where there are few cold chain facilities for moving vaccines to the field.

Progress in non-living oral vaccine development has, in general, been painfully slow. Although progress in our understanding of basic immunology has been spectacular in recent years the same does not apply to mucosal immunology. Although many papers have been published which describe the layout of mucosal immune systems and describe responses to antigens at mucosal surfaces there has been little success in tying these observations into differentiating between passive responses and protective immunity. Part of the problem lies in the inherent difficulties, especially in humans, of monitoring local

versus systemic immune responses. Another underlying problem is that the mucosal immune system has apparently evolved to avoid potentially harmful responses to dietary antigens. There is considerable evidence that repeated administration of large amounts of antigens orally can tolerize individuals.

Many papers describe immune responses to mucosally delivered antigens. Often however, the antigens are delivered in large or repeated doses or are poorly defined in molecular terms. Very few pure antigens have been described which are able to provoke strong local or systemic immune responses, when presented in small amounts in one or two doses to mucosal surfaces. This may in part be due to the harsh conditions antigens encounter. For example, when antigens enter the body through the oral route they have to cross the acidic stomach barrier and survive attack by digestive enzymes before they are sampled by immune cells in the gut. Clearly, most antigens will not survive this route intact. One approach to improving the oral delivery of antigens is to encapsulate them in materials that are acid resistant but biodegradable. Microbeads based on biodegradable materials have been used to encapsulate antigens such as ovalbumin to improve their oral delivery but still often high levels of material are required and practical vaccination is not yet available (O'Hagan, 1990). Perhaps an alternative and complementary approach is to look at the few known examples of effective mucosal immunogens in detail in the hope that lessons might be learnt for designing novel, non-living mucosal immunogens. A second point might be to look at the choice of route for mucosal delivery. Oral vaccination is inherently difficult to achieve reproducibly in the laboratory with pure proteins without using neutralization of stomach acids or gavage needles. A simpler way to reach mucosal surfaces is to intranasally vaccinate, particularly in small experimental models. An antigen might have more chance of reaching a mucosal surface intact in the upper respiratory tract than in the gut.

By far the best-characterized mucosal immunogens are members of the cholera enterotoxin (CT) family of molecules, including the *E. coli* heat-labile enterotoxin (LT). These molecules are highly immunogenic when delivered to mucosal surfaces and stimulate both local secretory and systemic responses. Further, it has been shown that microgram amounts of CT and LT can act as mucosal adjuvants, stimulating immune responses to co-administered antigens that are normally poorly immunogenic (Elson & Ealading, 1984; Holmgren & Czerkinsky, 1992). The structure of these toxins is known in some detail as the crystal structure has been determined for LT (Sixma *et al.*, 1991). The toxins are composed of two subunits. Subunit A is a single polypeptide, sometimes nicked, which has ADP-ribosyltransferase activity. The B subunit is a pentamer of five identical subunits tightly bound in the form of a ring-like structure. The B subunit is able to bind gangliosides located at the surface of eukaryotic cells and act as a delivery system of the highly toxic A subunit. The B subunit itself is non-toxic but is highly immunogenic.

CT is a highly toxic molecule in humans with ingestion of as little as 4 µg causing severe diarrhoea with up to 20 litres of stool within 48 h (Levine *et al.*, 1983). Although the mechanism of toxin action requires the intracellularization of the A subunit there may be much we do not understand about the toxin activity at the whole gut level. CT is believed to cause diarrhoea by stimulating intracellular cAMP in enterocytes but there is a suggestion that there might be neuronal involvement. CT is not as toxic for small mammals such as mice although it has been shown to be highly immunogenic in these animals when delivered orally (Elson & Ealading, 1994). Extensive experimentation has shown that CT and LT are potent mucosal immunogens with single doses of microgram quantities stimulating both local and systemic responses. The properties of the B subunits of these toxins are more controversial (Wilson *et al.*, 1993; Lycke & Holmgren, 1986). Experiments using the B subunit purified from toxigenic *Vibrio cholerae* have suggested that the B subunit is also a potent mucosal immunogen in humans. Work by Holmgren and colleagues, initially in volunteers and later in the field showed that a vaccine containing CT-B and high levels of inactivated whole *V. cholerae* cells could be safely used to orally vaccinate individuals and stimulate anti-CT-B responses (Svennerholm & Holmgren, 1986). Following oral administration of several doses the inactivated vaccine was shown to offer some protection against cholera and even diarrhoea caused by toxigenic *E. coli*. The group went on to develop an *E. coli*-based vaccine using a similar approach (Ahren *et al.*, 1993).

Later studies in volunteers by Holmgren's group and by George Griffin and David Lewis at St George's Hospital further defined the immunogenicity of the Holmgren vaccine (Lewis *et al.*, 1991). Lewis fed the vaccine, which contains about 1 mg of B-subunit and inactivated whole *V. cholerae* cells, to volunteers and carefully monitored the appearance of anti-CT-B antibodies in the serum (using ELISA) and antibody-producing cells (using ELISPOT) in the peripheral blood of vaccinees on selected days after vaccination in order to build up a temporal picture of the anti-CT immune response. A number of points became clear. After a single dose of the vaccine high levels of anti-CT-B IgA and IgG appeared in the sera. Antibody-producing cells also appeared in the blood in a reproducible fashion that was comparable between different volunteers. A wave of cells was detected for several days after vaccination which peaked on day seven. These peripheral cells could represent in part a population of lymphocytes trafficking between different mucosal surfaces during a maturation cycle, although this was not proven. Vaccinees who received a second dose of vaccine clearly exhibited a boosting response within some individuals up to 25% of the detectable circulating IgA-producing B lymphocytes making anti CT-B antibodies. These studies and later studies characterizing T-cell responses (Lewis *et al.*, 1993) clearly illustrate the potency of this mode of oral immunization.

We have shown using murine models that CT-B (unpublished) and LT-B (Lipscombe *et al.*, 1991) are effective mucosal immunogens when delivered intranasally as well

as orally. The intranasal route is a comparatively easy way of delivering antigens to mucosal surfaces, avoiding the requirement to pass the acidic stomach barrier. Intranasal immunization may be a simple option for characterizing and identifying novel mucosal immunogens. Eventually, it may be an option worth considering for the practical delivery of antigens in humans.

Why is CT such an effective mucosal immunogen? At present the answers to this question are unclear, although there are a number of properties associated with CT that might contribute. CT is able to bind to eukaryotic cells. This targeting of the protein may play an important role in presentation to gut-associated immune cells. CT is also resistant to protease activity, which may help it survive in the gut and at mucosal surfaces. CT has been reported to have a variety of effects on immune cells (Bromander *et al.*, 1991; Maghazachi, 1992). There is confusion in the literature about the properties of CT compared to CT-B. It is generally accepted that both CT and CT-B can induce immune responses when delivered to mucosal surfaces in humans although there is debate about whether CT-B is an effective mucosal immunogen in small animals such as mice. Certainly, many groups believe that CT, but not CT-B, can act as a mucosal adjuvant for bystander antigens (Wilson *et al.*, 1993). A potential source of confusion is that the CT-B reagent that is normally bought from companies is purified from toxigenic *V. cholerae* and as a consequence may be spiked with low, but biologically significant levels of CT (see O'Hagan, 1990). The use of antigen highly purified from recombinant hosts expressing either the holotoxins, engineered mutants of the toxins, or B subunit alone is required to clarify the situation. Lycke *et al.* (1992) recently described a study using a mutant form of LT harbouring a mutation which abolished ADP-ribosyltransferase activity but not ganglioside binding. They found that such mutants were poor mucosal adjuvants compared to wild-type toxin (Lycke *et al.*, 1992). We have recently embarked on experiments with highly purified derivatives of LT which have been mutated in key functions using site-directed mutagenesis (G. Douce, R. Rappuoli, M. Pizza & G. Dougan, unpublished results). Preliminary studies indicate that mutants can be obtained which are non-toxic but are still highly immunogenic. Such studies might help us piece together why these proteins are such potent mucosal immunogens. The identification of any non-toxic mucosal adjuvant would itself be a tremendous step forward for oral vaccine development.

Many groups have attempted to utilize the potent immunogenic properties of CT-B and LT-B by constructing systems for expressing fusion proteins composed of CT-B or LT-B and a heterologous antigen or epitope (see Wu & Russell, 1993). We developed a simple expression vector for creating fusions to LT (Lipscombe *et al.*, 1991). This approach has proved to be universally disappointing to date as the immune response to the carrier normally far outstrips the response to the carried epitope or antigen. However, this idea of non-living mucosal carriers might be more readily exploited with alternative mucosal immunogens.

Other non-living mucosal immunogens

What other examples are there of efficient non-living mucosal immunogens and do they exhibit common properties? The ability to bind mammalian cells may be one characteristic along with intrinsic resistance to proteases (see Fu *et al.*, 1993). We have carried out studies using the murine intranasal immunization model to screen for proteins which could induce systemic or local responses, particularly using proteins derived from pathogens. Using this system we were able to identify some proteins that could induce responses when administered in quantities of around 20 µg per dose. Examples of these are attachment factors of *Bordetella pertussis* including Fimbrial Haemagglutinin (FHA) and pertactin (Shahin *et al.*, 1992; Roberts *et al.*, 1993). Both FHA and pertactin can be used to protect mice using intranasal vaccination against a virulent aerosol challenge of *B. pertussis*. However, these proteins are not as immunogenic as CT-B or LT-B. We are currently constructing derivatives of these proteins in an attempt to define the regions required for mucosal immunogenicity.

Future directions

It is clear that we still have much to learn about the mucosal delivery of antigens in terms of vaccine development. The use of live delivery systems has been given new impetus by the use of recombinant DNA technology. The exciting developments are likely to involve the evaluation of heterologous proteins in carriers such as *Salmonella* in volunteers. Experiments in volunteers are already underway and early indications are that these studies are going to dramatically improve our understanding of human immune responses to infectious agents. This will be particularly true for cellular responses. The practical aims of this work will be to construct safely attenuated strains that can be used to induce protection against several diseases with a single oral dose. The main constraints on the pace of development are likely to be problems associated with vaccine stability and also considerations for the environmental release of recombinant bacteria.

Progress with non-living delivery systems is likely to come on several fronts. By studying the properties, in a comparative manner, of different mucosal immunogens, we may be able to identify the key properties associated with mucosal immunogenicity. If this is possible then we may be able to engineer new molecules that are mucosal immunogens. An approach would be to engineer features into protective antigens in order to improve their mucosal immunogenicity. This is likely to be a more long-term aim. The identification of non-toxic mucosal adjuvants is also likely to be of vital significance. Any novel mucosal antigens are likely to be incorporated into improved delivery systems which will offer protection of material on its way to mucosal surfaces and ideally would assist in targeting to gut-associated lymphoid tissues. An alternative avenue of research involves continuing studies on the basic immunology of the mucosal immune system. Can we learn to turn on mucosal-associated effector systems

more efficiently using parenteral rather than mucosal presentation? Already there are indications that this might be possible. The future certainly looks exciting.

I would like to dedicate this lecture to the following people. Derek Pickard for his marvellous contribution to this work, Dr Pavel Novotny for his unselfish and often unheralded scientific achievements and Dr Carlos Hormaeche, as a perfect example of an honest and open scientific collaborator.

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