The Molecular Basis of *Haemophilus influenzae* Virulence

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A major milestone in medicine was the discovery that germs cause disease and that particular clinical syndromes may be characteristic of infections caused by specific micro-organisms. Entrenched as is this notion in clinical practice, it is sobering to recall that its discovery is barely a century old. The criteria for causality of an infection by a particular microbe are embodied in the postulates of Robert Koch; an invariable association of the microbe and the disease in question and that the microbe should cause an identical infection when reintroduced into a susceptible animal after first being cultured through several generations *in vitro*. As with many shibboleths, particularly when all criteria are not rigorously applied, embarrassments occur. So it was that Gram-negative rods were seen so frequently in the sputum of individuals with influenzal pneumonia in the pandemic of 1889–92 that these bacilli were considered to be its cause and were named *Haemophilus influenzae*[1]. It was not until 30 years later that Andrewes discovered that influenza was caused by a virus[2], by which time the appellation of *Haemophilus influenzae* was well established. However, *H. influenzae* is no idle pretender; it causes a wide spectrum of infections, especially in childhood, including meningitis, septic arthritis, pneumonia and empyema, otitis media, epiglottitis and cellulitis. Of these, meningitis is of particular importance because of its potential, even with appropriate supportive and antibiotic treatment, to cause lasting neurological damage[3]. Deafness, convulsions, mental retardation, hemiplegia and other, sometimes subtle, neurological deficits have been recognised. The occurrence of these apparently permanent neurological sequelae in a substantial proportion of survivors of *H. influenzae* meningitis is partly attributable to the fact that this disease occurs almost exclusively in children aged less than four years and, in particular, in infants and children of less than two years in whom the brain is in a critical phase of development. The impact of *H. influenzae* meningitis has been well studied in the USA where approximately 10,000 cases occur each year[4]. Table 1 summarises the morbidity and mortality from this disease based on studies from North America. Attack rates in the United Kingdom may be somewhat less, but recent data[5] indicate that the incidence of *H. influenzae* meningitis is not dissimilar from that observed in the USA and the few studies which have been done to assess its impact on English children indicate that it is equally serious[6].

Table 1. Long-term neurological residua from *H. influenzae* meningitis.

	Sell[22] (n = 86) %	Feigin[23] (n = 86) %
Significant residua[1]	30.2	8.0
Possible residua[2]	14.0	28.0
None detected	43.0	64.0

[1]IQ < 70, seizures, hearing loss (severe), motor deficits, or partial blindness
[2]IQ 70–90, hearing loss (mild), speech problems, or behaviour problems

Our understanding of the biochemical basis of the pathogenicity of *H. influenzae* stems from observations made more than 50 years ago; strains of *H. influenzae* that cause meningitis are almost always encapsulated[7]. In fact, *H. influenzae* may make any one of six chemically distinct polysaccharide capsules and the antigenic differences mediated by these capsules is the basis of serotyping, i.e. types a-f or non-typeable. It is remarkable, however, that the majority of cases of meningitis are caused by type b strains whereas other types or non-capsulated strains rarely cause meningitis. A further long-standing observation is that susceptibility to *H. influenzae* meningitis is strongly correlated with absence of serum antibodies to type b capsule[8,9]. This, with the experimental data[10,11], makes the circumstantial evidence that the b capsule is a major virulence factor extremely strong. Based on these facts, it seemed entirely logical to attempt prevention of *H. influenzae* meningitis by using the purified type b capsular polysaccharide, polyribosylribitol phosphate (PRP) as a vaccine. Indeed, a similar approach had been used to prevent serious infections caused by encapsulated *Streptococcus pneumoniae* and *Neisseria meningitidis*[12,13]. However, PRP proved to be insufficiently immunogenic to raise protective levels of serum antibodies in young children in whom the highest incidence of *H. influenzae* meningitis occurs[14].

The failure of this logical approach to immunisation suggested the need for developing alternative strategies for prevention. These depend upon more thorough understanding of how host and microbial determinants of

the infection determine the pathogenesis of meningitis. Thus, the aim of studies conducted in my laboratory—formerly at the Johns Hopkins University and more recently in Oxford—is to understand the molecular basis of *H. influenzae* pathogenicity. Virulence is a complex biological phenomenon and a *sine qua non* for its study is the availability of a biologically relevant model infection. For if one says that a bacterium is virulent, one must ask for whom? Conversely, from the host's point of view, if an animal is susceptible to a micro-organism one must ask to what? Ideally, one should strive to understand these questions at the molecular level. It has been shown that infant rats provide a biologically relevant model of *H. influenzae* meningitis[15]. Based on several years of studies, many features of the pathogenesis of *H. Influenzae* meningitis have been elucidated. If virulent type b organisms—e.g. strains cultured from the CSF of children with *H. influenzae* meningitis—are inoculated intranasally, the nasopharynx becomes heavily colonised and, within hours of the challenge, organisms enter the blood stream. Type b organisms are able to evade clearance by the spleen, liver and other reticulo-endothelial organs which mediate the clearance of blood-borne bacteria. If sufficient organisms circulate for a sufficient duration, the probability of meningitis is high. In fact, a striking correlation exists between the magnitude of bacteraemia and the occurrence of meningitis. The implications of this latter finding, borne out by experimental observations, is that a major determinant of meningeal invasion resides in those microbial factors which confer upon *H. influenzae* resistance to phagocytic clearance from the blood stream. However, it needs to be emphasised that isolating one stage in the pathogenesis and assigning to it a pivotal role is over-simplistic, for these events must be placed into an appropriate biological context. Thus, virulence is dependent on the interaction of many different bacterial genes with those of the host. Indeed, those bacterial genes which determine bloodstream survival are not necessarily the same as those which mediate lodgement in the nasopharynx, or determine the translocation of organisms from the nasopharyngeal lumen to the blood, or the invasive process which results in penetration of the blood/central nervous system barrier. In fact, several putative virulence factors have been identified (Table 2) and others probably exist of which we are not yet aware. The challenge is to sort out the role of each and a logical way to do this is by taking a genetic approach. If the genes or sets of genes which determine the expression of these putative virulence factors are identified, strains can be constructed which are sufficient or deficient in expressing one or more of these attributes. This genetic manipulation, i.e. the construction of isogenic strains, has become a practical possibility through the availability of cloning and recombinant DNA techniques. This strategy is outlined in Fig. 1. One constructs a gene library which must be searched to isolate recombinant phage which possesses one or more genes relevant to the expression of a particular virulence factor. When the relevant cloned DNA has been characterised, it can be introduced in modified or unmodified form into haemophilus by DNA transformation. This provides a method for constructing isogenic strains that can be used for virulence studies, the results of which should provide an unambiguous indication of the role of a particular gene or set of genes in determining virulence.

Table 2. Putative determinants of virulence potential in *H. influenzae*.

Capsular polysaccharide
Lipopolysaccharide
Outer membrane proteins
Pilus proteins
IgA_1 proteases

Now this broad approach is relatively simple when dealing with virulence mediated by an enzyme or perhaps a toxin since these polypeptides are usually coded for by only one or two genes; such is the case for the sub-unit pilin, individual outer membrane proteins and the $IgAl_1$ proteases of *Haemophilus influenzae*. Thus the gene library can be searched using a radio-labelled probe to identify DNA clones expressing the protein. This was the approach used to clone a gene for an IgA_1, protease of *H. influenzae*[16]. However, the situation is much more complicated when one is attempting to clone the genes for expression of capsular polysaccharide or lipopolysaccharide. A logical approach to identifying these genes would need to be based upon an understanding of the enzymes controlling their biosynthesis, cytoplasmic transport, polymerisation and surface assembly. Furthermore, the coordinated expression of these genes is likely to be under the influence of complex expression mechanisms, i.e. bacterial surface antigens are prone to be both switched on and off as well as to exhibit antigenic variation[17]. A further fact which must be taken into account is the location and organisation of the genes on either chromosomal or extra-chromosomal DNA. The major possibilities are considered in Fig. 2. Based on the results of transformation experiments it seemed clear that only the first two possibilities were likely. High molecular weight DNA was extracted from a virulent type b strain and used as the donor for transformation. The average size of the donor DNA in such an experiment was approximately 40,000 base pairs. By carrying out the transformation under conditions in which donor DNA was limiting, transformants resulted from the uptake of a single molecule of DNA. Thus, it could be concluded that genes necessary for b capsule expression were linked.

This approach was used to generate a series of transformants which have provided a series of genetically similar strains for comparative virulence studies. Experimental infection of rats showed that the type b transformant was significantly more virulent as evidenced by both the incidence of bacteraemia and the quantity of organisms recovered from the blood. Furthermore, each of the transformants, representing the different capsular serotypes, had the potential to reach the blood stream, thus indicating that the greater virulence of the type b transformant resided in its greater ability to survive in the bloodstream. Since meningitis is a function of magnitude

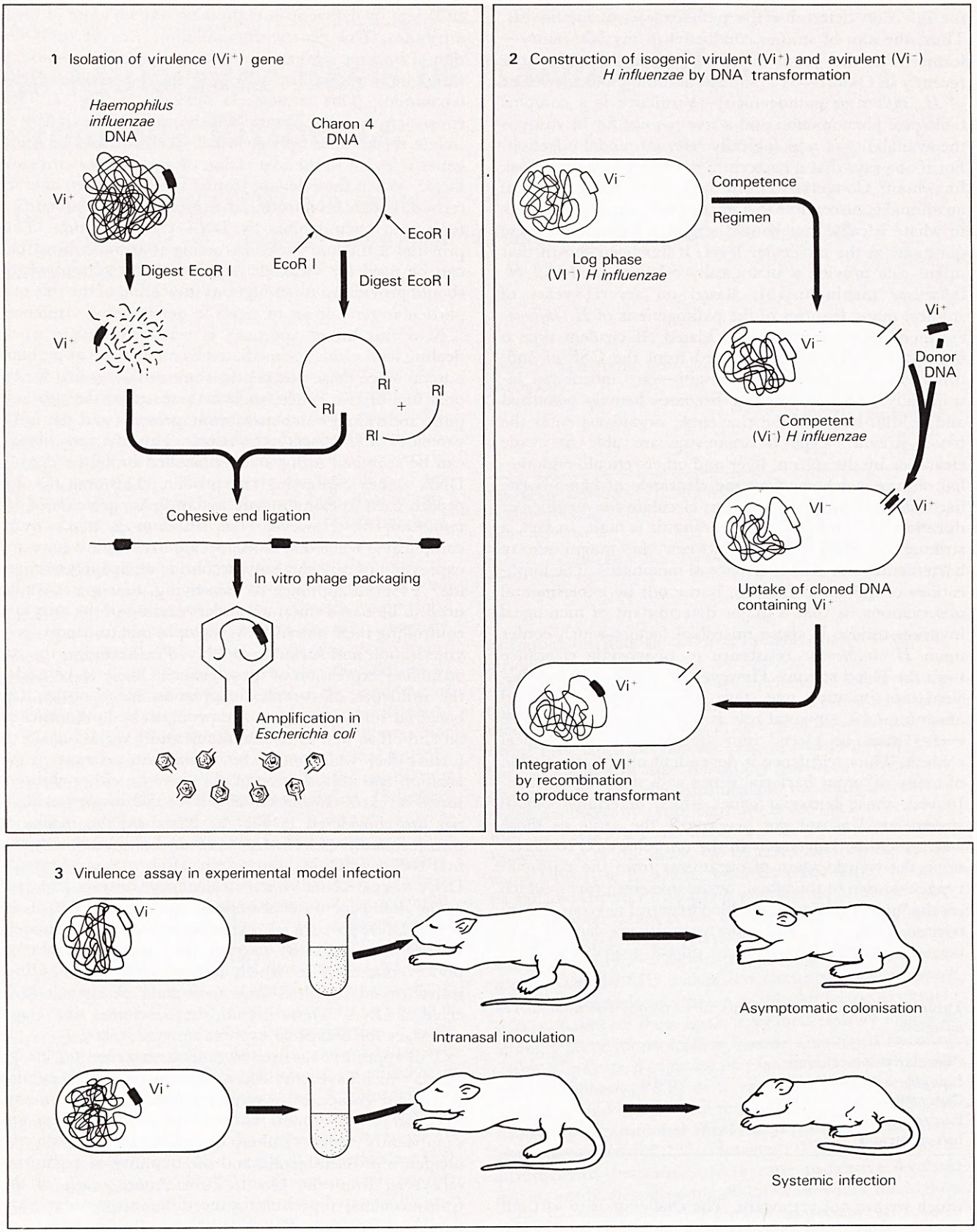

Fig. 1. *General scheme for studying pathogenicity of* H. influenzae. *Fragments of chromosomal DNA are cloned into a suitable vector to isolate virulence genes (Panel 1). These genes can be introduced by DNA transformation into* H. influenzae *strains which lack the specific virulence determinant (Panel 2). Transformed and untransformed* H. influenzae *are then compared in a suitable animal model, e.g. infant rats (Panel 3) to allow an unambiguous analysis of the role of specific genes in pathogenicity.*

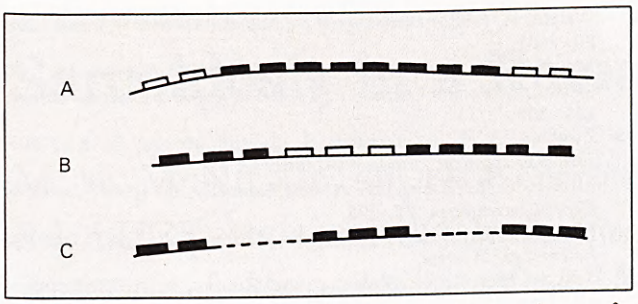

Fig. 2. *Hypothetical possibilities for arrangement of genes for type b capsule expression. It is assumed that several genes (the choice of 8 is arbitrary and speculative) are required for the biosynthesis, transport, polymerisation and surface assembly of the ribosyl-ribitol phosphate polymer. These might be arranged: A. as a linear cluster of genes; B. closely linked, but with two or more clusters interspersed by genes unrelated to capsule expression; C. widely dispersed so that genes are not linked.*

of bacteraemia[18], the data suggested that acquisition of type b capsule plays a critical role. But there is reason for caution in this interpretation; did transformation result in the acquisition of genes other than those coding for type b capsule? To examine this question, we studied the outer membrane proteins (OMP) and lipopolysaccharide (LPS) phenotypes of the transformants, these being two obvious cell surface antigens that might contribute to pathogenicity. Whereas the OMP of the transformants were similar, the LPS phenotypes were distinctly different[19]. Thus, transformation apparently caused altered expression of not just capsule, but also LPS.

This experiment illustrates why alternative approaches are necessary to examine critically the independent role of capsule and LPS. This is not straightforward, since both LPS and capsule expression are each the function of several genes.

To analyse the contribution of type b polysaccharide, the approach was as follows: capsulated strains produce non-capsulated, single-step mutants at rather high frequency (approximately 0.1 per cent). From a sectored colony, we obtained a stable capsule-deficient mutant (Sec-1) and then screened our lambda gene library for recombinant phage which possessed DNA inserts which would transform this capsule-deficient recipient to capsule production. Thus, some lambda clones had the ability to restore the typical, iridescent colonial phenotype to the capsule-deficient recipient. One such recombinant, designated Charon 4:48[20] consisted of a 13.4 kb insert of haemophilus DNA. It was mapped using restriction enzymes and shown to have two Eco RI fragments of 9 and 4.4 kb. These were subcloned into pBR322 and used as donor DNA for transformation of the capsule-deficient strain, Sec-1. In this manner, we were able to show that Sec-1 has a mutation which maps to a 1 kb region within a 4.4 kb Eco RI fragment[21]. By Southern hybridisation, it was also shown that the mutation involves either a very small deletion or rearrangement in this 1 kb region. Thus a comparison of the virulence of these two strains is informative, since they apparently differ in the expression of a single gene, a gene which is necessary but not sufficient for type b capsule expression. The type b strain was virulent, whereas Sec-1 was not. The result was not unexpected, but provided rigorous proof of the essential role of b capsule in virulence expression.

It has also become apparent that altered expression of a cell wall antigen (most probably LPS) is also critical to virulence expression. To search our library for a clone involved in LPS expression, we made use of the fact that LPS mutants often have an opaque phenotype; a lambda clone from our library, designated Charon 4:I69, was isolated. This clone contains an appx. 10 kb Eco RI fragment which was capable of producing a transformant (Rd/b$^+$/I69) which retains full capsule expression, no definite alteration in OMP but a definite change in LPS phenotype. When the parent strain and its I69 transformant were compared in virulence experiments, the transformant failed to produce bacteraemia and meningitis whereas the parent strain was fully virulent[19]. One difference between the parent and transformed strains is that the I69 transformant is rapidly cleared from the bloodstream following intravenous inoculation, whereas the untransformed parent survives very efficiently. Thus, expression of both type b capsule and LPS appear to be critical, independent determinants of the bloodstream survival of *H. influenzae*.

These experiments indicate an approach to the analysis of microbial virulence and how this approach could amplify our basic understanding of the molecular mechanisms of bacterial virulence. Ultimately, one hopes that this approach will facilitate the development of novel approaches to prevention of important infections. In a sense, as Stanley Falkow has observed, Koch's postulates can be modified so as to have a molecular ring to them. For we should now aim to isolate virulence genes, amplify them in their respective vectors, re-insert them into an avirulent strain and demonstrate the re-acquisition of pathogenicity and the potential of that organism to produce classic disease.

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highly of the medical profession[8], few of his illustrations have medical associations. He made a frontispiece for a book on diseases of horses, by another Wilkinson[9], and Bewick's apprentices engraved the illustrations for *A New Family Herbal* by Thornton, in 1810[10].

In commissioning a local engraver to provide the frontispiece for his small treatise, Wilkinson was not in a position to appreciate the full significance of the illustration; nor was he able to anticipate that an extract from willow-bark provided the incentive for the synthesis, some 70 years later, of one of the most widely used of all therapeutic agents, the salicylates.

George Wilkinson's small book, with its frontispiece by Thomas Bewick, is available in the College library. It contains two special features: a dedication inscribed by the author to the President and Fellows, and the insertion of some pressed broad-leafed willow leaves. Bewick's works are well represented in the library; there are two separate editions of both *A General History of Quadrupeds* and *The History of British Birds*. Thornton's *New Family Herbal* (1810), the illustrations for which were engraved by Bewick's apprentices, is also included.

In the College collection is the well illustrated and handsome folio first edition of Pierre Pomet's *Histoire Générale des Drogues* (1694). This first French edition was translated into English (1712) and published with the title *Compleat History of Druggs;* Pomet was described as the 'chief druggist to the late King Lewis (sic) XIV'. The anonymous translator dedicated the work to Sir Hans Sloane. It became a popular work and is often regarded as the first comprehensive materia medica in English. The College's English edition (1748) is the fourth, in which the editorial approach is dubious, for it had been amended and rendered 'less obscure' by the self-styled 'Sir' John Hill, opportunist and quack, a man referred to by Dr Johnson as ingenious but having no veracity.

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