Current topic**Virulence genes and prevention of *Haemophilus influenzae* infections**

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The bacterium *Haemophilus influenzae* causes a wide spectrum of important childhood diseases that includes meningitis, epiglottitis, cellulitis, acute pneumonitis, septic arthritis, and otitis media. Meningitis, the commonest of the systemic infections, in addition to being life threatening, is of particular importance to paediatricians because the damage it causes to the developing brain is often permanent. *H influenzae* is a major cause of pyogenic meningitis in childhood throughout the world, and occurs in about one child in every thousand, usually within three years of birth. Although the availability of antibiotics has decreased mortality dramatically (from greater than 90% to less than 10%), the occurrence of central nervous system damage among survivors has not declined substantially during the past three decades. Deafness, convulsions, mental retardation, hemiplegia, impaired language skills, and other neurological deficits are among the common sequelae,¹ and the emergence of antibiotic resistant strains of *H influenzae* threatens to compromise even further the limited efficacy of current treatments.

A different example of *H influenzae* pathogenicity is its role in causing about 20% of cases of otitis media in childhood. Although this is less dramatic than meningitis, it is extremely common and is an important cause of hearing impairment leading to school failure. A more controversial problem is the role of *H influenzae* as a lower respiratory tract pathogen; there is now a growing body of evidence that it is one of the commonest causes of severe pneumonia in infants in underdeveloped countries.² There can be no doubt that the successful prevention of *H influenzae* infections would represent a major step forward. To this end, substantial gains are now in evidence.

Recent progress builds upon two pivotal observations made more than 50 years ago. The first, contributed by Margaret Pittman, was the recognition that the strains of *H influenzae* that cause meningitis (as well as most other bacteraemic

infections) are encapsulated.³ *H influenzae* may make any one of six chemically and antigenically distinct polysaccharide capsules (designated a-f), but strains expressing type b antigen account for most serious infections. The second important observation was that serum factors (later identified as antibodies) with specific activity against the type b antigen are critical in host defence against systemic *H influenzae* infections.⁴ Given these facts, it is reasonable to ask what is so important about the type b capsule of *H influenzae*, how does it differ from the five other polysaccharide capsules, and to what extent other surface antigens, such as outer membrane proteins and lipopolysaccharide, modulate *H influenzae* virulence or serve as targets for the lethal effects of host immune responses. Answers to these questions should provide rational approaches to the prevention of *H influenzae* infections.

The analysis of microbial virulence has depended traditionally on identifying characteristics of microbes that correlate with their potential for pathogenicity (a familiar example is the use of the coagulase test to identify virulent strains of staphylococci). This sort of empirical correlation, despite its practical use, does not necessarily enhance understanding of the mechanisms of microbial injury or of host immunity. This is because these phenotypic markers may correlate with virulence potential without necessarily being instrumental in the pathogenic process. To determine whether or not this is so, one must compare the virulence of isogenic variants, that is strains which are identical except that they are either sufficient or deficient in the gene or genes required for the expression of a particular virulence determinant. This can now be achieved through the application of recently developed techniques of genetic manipulation of bacteria. Indeed, the application of recombinant DNA technology to the study of microbes is revolutionising the study of the epidemiology, diagnosis, treatment, and prevention of infectious diseases. As a result, basic mechanisms of microbial virulence are

now immensely more accessible, and over the next several years infectious diseases research should prove as exciting a field as at any time since the role of microbes as causes of disease was discovered just over a century ago.

As in other areas of medicine, the application of this new technology has moved quickly from the basic science laboratory into the clinical setting, and paediatricians are therefore being encouraged (even urged!) to ensure that they possess a modicum of 'literacy' in molecular genetics. Only a modest investment of time is required to capture the basic principles and a number of admirable, succinct essays have been published in general medical journals which provide the practising physician with as simple and rewarding a task as possible. Thus primed, the occasional articles published in this and

other paediatric journals which require a grasp of recombinant DNA techniques will not daunt those with a little determination and patience, even though it may seem that some authors are determined to obscure their essential messages in the sophistry of this new and exciting field!

A general strategy used in tackling the challenge of *H influenzae* is illustrated in the Figure. This bacterium possesses a single, circular chromosome consisting of almost two million paired nucleotides. If we consider the type b capsule, its expression must depend on genes that control the biosynthesis, transport, polymerisation, and surface assembly of the polysaccharide. To identify these genes, chromosomal DNA from a representative strain is cut into hundreds of pieces using restriction enzymes (Figure (a)). These constituent pieces of DNA are

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

then introduced into a vector, for example the modified bacteriophage Charon 4, using appropriate (commercially available) ligation and packaging steps. Since the recombinants contain random inserts of *H influenzae* DNA, the result is a library of fragments cloned into the vector. If sufficient recombinants are generated, then the entire genome will be represented. The recombinants, each containing passenger DNA, are allowed to infect their natural host, the bacterium *Escherichia coli*, thus allowing the numbers of each of the representative recombinants to be replicated many fold. The next step is to identify the relevant recombinants with inserts of haemophilus DNA required for the expression of a particular virulence determinant. Several approaches could be useful here. One obvious ploy is the use of antibody to identify clones expressing a particular protein; another strategy is to search the library for cloned DNA capable of restoring virulence expression to a strain lacking this capability because of a mutation. This requires a technique for inserting haemophilus DNA into the avirulent mutant, a need conveniently accomplished using DNA transformation. Transformation is one of the natural mechanisms of genetic exchange characteristic of *H influenzae*. The essentials of transformation are illustrated in the Figure (b).

(1) A defined piece of DNA, which might be a virulence gene (Vi+), is added to a culture of *H influenzae* that has been incubated in a special media to make the bacteria competent for DNA uptake. These competent bacterial cells take up this DNA, thus resulting in the acquisition of novel DNA and an altered genotype. This sets up the final step (Figure (c)), which is to compare the virulence of the original and transformed strains in infant rats, an accepted model of the human infection, so that differences in virulence potential can then be unambiguously assigned to the cloned DNA. Furthermore, by varying the route of inoculation or modulating the immune status of the animal, much can be learned about the stages of the infection at which a particular virulence determinant exerts its effect. This in turn could lead to the recognition of key host determinants such as tissue receptors for bacterial adhesion, local or serum antibodies.

Using this type of approach, a library of *H influenzae* DNA has been successfully searched for genes involved in type b capsule expression.⁵ The isolation of these genes has provided unambiguous evidence of the critical role of type b capsule in virulence since a mutation in one of the genes necessary for its expression results in loss of invasiveness and the correction of the mutation by transformation with cloned DNA restores the fully virulent phenotype. This result was anticipated since

an enormous body of circumstantial evidence already existed implicating the type b capsule as a major factor in preventing the efficient ingestion of *H influenzae* by phagocytic cells. Experiments along similar lines are now establishing the role of other virulence genes, such as those needed for the expression of other proteins and lipopolysaccharides that make up the bacterial cell envelope. Also, strains of *H influenzae* with and without an enzyme known to cleave human immunoglobulin have been constructed.⁶ Thus, a relatively comprehensive analysis of the unique contribution of different virulence determinants is now feasible.

An exciting bonus of this approach is the use of the cloned virulence genes as probes to analyse clinical isolates from all over the world using the simple but enormously powerful technique of Southern hybridisation. Readers of this journal have been recently introduced to the excitement that has been generated in the field of human genetics through the identification of DNA polymorphisms for studying familial diseases such as Duchenne muscular dystrophy. The identification of restriction fragment length polymorphisms (RFLPs) that are closely linked to critical genes has proved spectacular since a successful search in the case of Huntington's chorea was initiated without any prior knowledge of the location of the affected gene or its mechanism of causing disease. These ideas seem applicable to the problem of locating informative sequences in the bacterial genome. Fortunately, there are sequence repetitions in the region coding for the b capsule. Using a probe from this region, our laboratory has analysed more than 100 type b strains isolated from children with invasive infections from Europe, America, Africa, and Australasia. Three polymorphisms describe more than 95% of these isolates, each of which is highly associated with a characteristic outer membrane protein subtype.⁷ The recognition of a set of invariant characteristics among pathogenic strains, diverse both geographically and temporally, has important implications for prevention, since antigenic variation increases the complexity of developing successful immunisation strategies. The same kind of approach could reasonably be used to analyse unencapsulated strains responsible for otitis media or pneumonia. In either instance, the critical virulence genes, or sets of genes, are not known. The idea would be to identify one or more polymorphisms characteristic of the pathogenic, as opposed to random carrier strains, in the hope that these would be informative. The rationale for this approach is strengthened by the observation that for many pathogenic bacteria a limited number of genetically related virulent strains (presumably derived from common ancestors)

accounts for almost all those implicated in causing disease.⁸

The approach outlined above, or variations of it, offers an approach to understanding the fundamental determinants of *H influenzae* virulence, and is broadly applicable to other pathogenic micro-organisms. Meantime, paediatricians will have noted that the efforts of more than a decade of intensive research by several investigators based in the USA (supported energetically by the National Institute of Health) have culminated in the licensing of an *H influenzae* vaccine consisting of purified type b capsule—b-Capsa I.⁹ This is a welcome and exciting development. Furthermore, the vaccine is essentially free of any untoward reactions. But, b-Capsa I is imperfect since it offers solid protection only against type b infections occurring in children aged 2 years or more.¹⁰ This means that it will not prevent type b infections in the younger children, who represent the most important target population; it will be recalled that most cases of systemic *H influenzae* infections, including meningitis, occur in children less than 18 months of age. The vaccine is not expected to have any appreciable effect against *H influenzae* otitis media, irrespective of age, since more than 90% of these episodes are caused by unencapsulated strains of *H influenzae*. Similarly, with reference to the problem of lower respiratory infections in developing countries, it must be anticipated that the vaccine will have little impact on morbidity or mortality because these children are mostly too young to respond and many of the isolates are not of type b. On the optimistic side, however, the limited efficacy in preventing type b infections is not likely to last for very long. When type b capsular polysaccharide is conjugated to protein, both the quantitative and qualitative aspects of its immunogenicity are changed dramatically for the better.^{11 12} Large scale trials of immunogenicity and protective efficacy are already in progress for two of the candidate conjugate vaccines, and they are virtually free of any untoward reactions. I think it is entirely realistic to predict that type b infections will soon be preventable by immunisation but, as we know from the shameful

situation in the United Kingdom as regards measles immunisation, the availability of a vaccine is a far cry from that of delivering it to the target population. There is visible light at the end of the tunnel—but only just.

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