Microbes, molecules and man

The Mitchell Lecture 1992

ABSTRACT—Robert Koch, the discoverer of the tubercle bacillus, has had a seminal influence on the extraordinary progress in the field of infectious diseases in the past 100 years. Koch's postulates defined the germ theory of disease causation. They have now been confirmed and brought up to date by the application of molecular techniques. Developments in molecular genetics have helped in the elucidation of the pathogenesis of meningitis caused by *H influenzae* and the role of type b capsule as a determinant of bacterial virulence—and satisfied the requirements of Koch's molecular postulates. This new knowledge has contributed to the development of a successful immunoprophylactic strategy for eliminating Hib disease. Studies in Oxford over the past eight years have confirmed the effectiveness and safety of a routine immunisation programme for the UK.

This lecture was initiated by a gift of £500.00 in 1917 by Mr Mitchell (Fig 1), a businessman from Birmingham, who placed the gift in the hands of Sir Edward Malins, a distinguished obstetrician elected as a Fellow of the College in 1902. The College resolved that there should be a triennial lecture on tuberculosis, but in recent years the remit has been widened to include other infections.

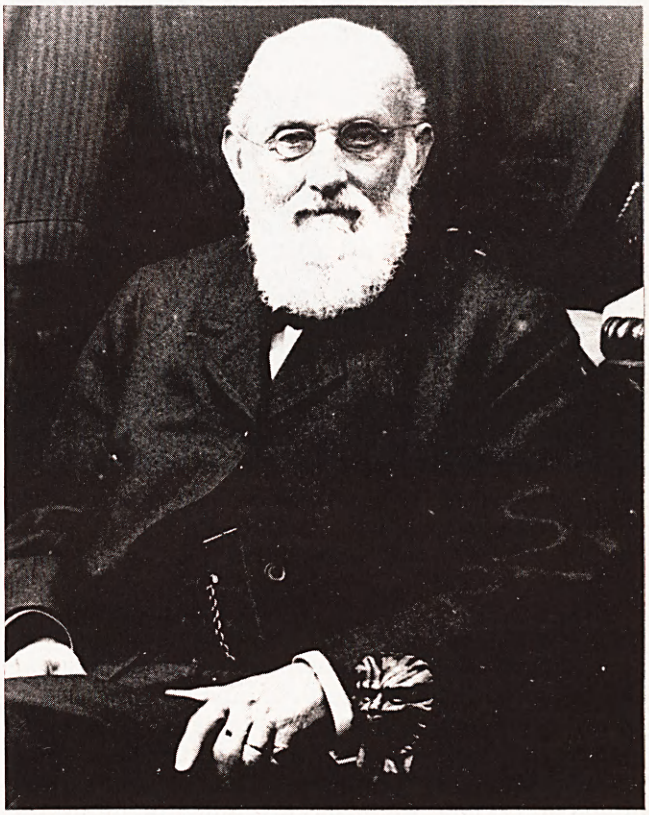


Fig 1. *Mr Mitchell. A Birmingham business man who in 1917 donated £500.00 to found the lectures (courtesy of the library of the RCP).*

Microbes

The most famous name in medicine in the first half of the 20th century was Robert Koch (Fig 2) who discovered the tubercle bacillus and was honoured by a Nobel prize in 1905. In 1890 bacteriology was a new science, barely two decades young. The idea that microbes were the cause of disease was highly controversial, but Koch was an indefatigable proponent of the germ theory of disease, the principles of which were embodied in the postulates that bear his name [1]. Koch's postulates have not proved infallible, especially in the context of diseases caused by slow viruses or in considering the role of microbes in the immune-compromised host. Nonetheless, the notion that microbes are the necessary, but not sufficient, cause of particular diseases represents one of the major advances in the history of medicine. The concept of 'necessary but not sufficient' in causation is important but appears not to have been recognised by such eminent minds as John Maddox (editor of *Nature*) and Peter Duesberg in their recent *tour de force* on the role of the human immunodeficiency virus and AIDS [2].

As we have moved from the golden age of microbiology to the era of molecular biology, Koch's postulates have undergone a major revision, a transition that has literally revolutionised science and is changing the world in which we live. How did this happen? Just after the First World War Dr Fred Griffith, a medical officer in the Ministry of Health in London, was working on the problem of pneumonia caused by the pneumococcus. What intrigued Griffith was the fact that a single sample of sputum from a pneumonia patient could harbour four or five different strains (capsular serotypes) of pneumococci. Curiously, Griffith seemed reluctant to believe that each strain had

E R MOXON, FRCP
Professor of Paediatrics, Oxford University

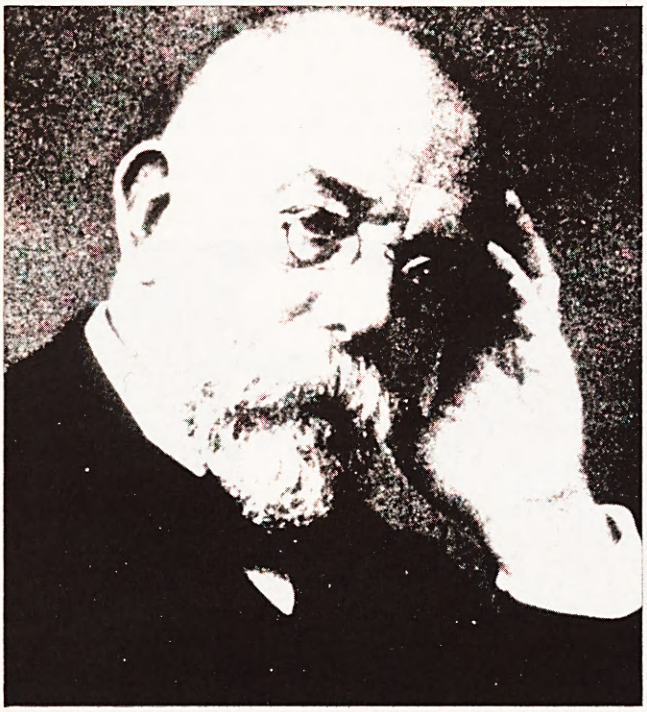


Fig 2. *Robert Koch who discovered the tubercle bacillus and in 1905 won the Nobel prize.*

been acquired as a separate transmission event; rather, he wondered whether pneumococci were undergoing changes of serotype as a result of immune pressures or other host-related influences, a thought provoked in part by the readiness with which, in the laboratory, pneumococci could be shown to lose or gain capsule production. These ruminations resulted in an historic experiment in which he hypothesised that killed encapsulated pneumococci could provide substances that would enable living capsule deficient pneumococci to be transformed to the encapsulated state. His findings were published in a 46 page report [3], and met with considerable scepticism. The doubts were soon diminished when Neufeld, working in the Robert Koch laboratory in Berlin, obtained identical results. The phenomenon of transformation had been discovered, but an explanation had to wait a further 20 years. Neither Griffith nor Neufeld got to the bottom of what they had discovered and there is a sad historical footnote; Griffith died in an air raid in 1941 and Neufeld died of starvation in war ravished Berlin in 1945.

But the experiment had not failed to arouse the intense curiosity of scientists in the Rockefeller laboratories in New York where a succession of young investigators took on the problem of solving the chemical nature of the 'transforming principle' under the aegis of Oswald Avery. The denouement is well known; the transforming principle turned out to be deoxyribonucleic acid, DNA [4], in this case part of a gene or genes required for capsule synthesis. What Griffith had stumbled upon was no less than the biochemical basis of heredity, the necessary but not sufficient basis of life itself. The structure of DNA was proposed in 1953 by Watson and Crick and suddenly Schrödinger's seemingly arrogant question captured in the title of his classic book *What is life?*, seemed less remote to scientists. The age of 'molecular biology' had truly arrived. In 1967, Hamilton Smith isolated and characterised the first restriction endonuclease, an enzyme that could recognise and cleave specific nucleic acid sequences [5] and this provided an essential tool with which Dan Nathans showed how genes could be mapped [6]; Boyer and Cohen pioneered the tools for recombinant DNA [7] to begin the era of what has come to be called the 'new genetics' [8]. The ability to manipulate the genes of pathogenic microbes is only one of the applications of recombinant DNA in the medical sciences but the study of infectious diseases has undergone a revolution. A molecular version of Koch's postulates, provided by Stanley Falkow [9], is as follows.

- The phenotypic property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
- Specific inactivation of the gene associated with the suspected virulence trait should lead to the measurable loss in pathogenicity or virulence.
- Revision or allelic replacement of the mutated gene should lead to restoration of pathogenicity.

In less formal terms, one can isolate a candidate virulence gene from a bacterium (or any microbe) through cloning, or these days using polymerase chain reaction, make virtually as many copies of the gene as are needed in a 'test tube', make mutations in the gene and insert the altered or wild-type gene into a bacterium to determine its function. The elegance of this approach is that one can compare defined variants that differ uniquely in the presence or absence of a single gene and see whether the gene in question can confer the ability to cause disease in a biologically relevant model.

I was working on the pathogenesis of bacterial meningitis at Johns Hopkins University in Baltimore when I heard Nathans' lecture on mapping the SV 40 viral genome. I learned for the first time of the first restriction enzyme, HindII isolated from *Haemophilus influenzae* type d for which Nathans, Smith and Arber received the 1978 Nobel Prize in Medicine and Physiology. Smith's laboratory, situated across the street from the hospital, was applying their brilliant new technology to unlocking the biochemical basis of transformation, one of the general genetic mechanisms used by bacteria to exchange DNA. Fortunately, Smith allowed me the use of some bench space to study the virulence genes of *H influenzae,* the most

common cause of pyogenic or purulent meningitis in young children. It is a global problem, the disease occurring in every country where adequate epidemiology and bacteriology allow the disease to be properly defined. The child shown in Fig 3 illustrates the severe opisthotonos characteristic of basal involvement in bacterial meningitis. Such an extreme clinical presentation would be rare in Europe or North America, but in many parts of the world disease of this severity occurs frequently; *H influenzae* affects about one in every five hundred children by their fifth birthday. Death and disability from *H influenzae* meningitis is a major problem. A gram stain of spinal fluid reveals numerous extracellular bacteria, short gram-negative coccobacillary organisms (Fig 4); there are also many inflammatory cells whose role is to ingest and kill microbes; but the striking feature is that virtually all the organisms are extracellular, indicating that the polymorphs are ineffective. This implies that the surface of the *H influenzae* organisms possesses properties that deter their binding and ingestion by phagocytes. The most important of these surface structures is capsular polysaccharide. In fact, *H influenzae* can make six chemically different polysaccharides but, remarkably, a single one of them, a polymer of alternating units of ribose and ribitol (designated PRP for polyribosylribitol phosphate), accounts for more than 97% of meningitis cases caused by *H influenzae*. In general, capsule functions as a virulence factor through impeding phagocytic ingestion and killing by complement.

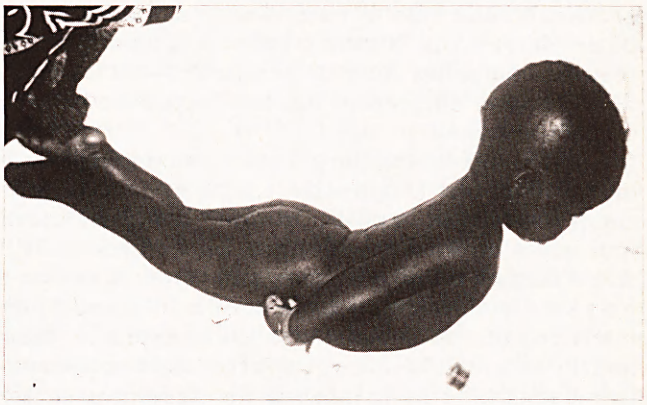


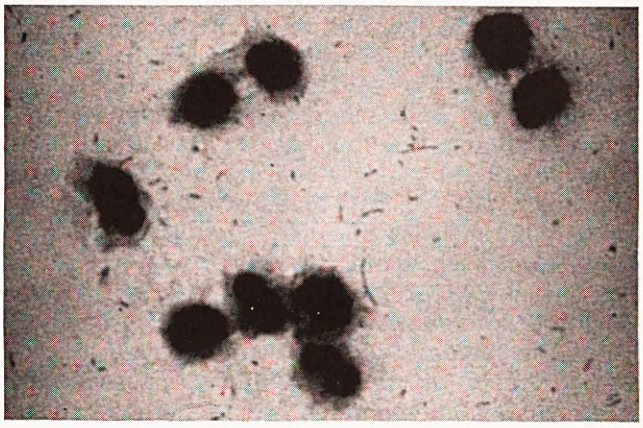
Fig 3. *The severe opisthotonos characteristic of basal involvement in bacterial meningitis is illustrated in this child.* (Photograph courtesy of Dr Charles Newton.)

Molecules

Given this information as background, my laboratory set out to use molecular genetics to answer two questions of relevance to *H influenzae* meningitis. First, knowing that type b strains of *H influenzae* (Hib) cause the majority of invasive diseases by this organism, what is the role of PRP in virulence? This is a little bit more complex than it might seem. The association of PRP and invasive Hib disease is a fact but this association could be explained by at least two contrasting hypotheses. One is that capsule made of PRP may itself confer virulence properties denied to the other capsules; the other that PRP may merely be a phenotypic marker, a factor present in strains of enhanced pathogenicity but itself not uniquely more capable to determine virulence than other capsular polysaccharides. Thus, in hypothesis one, PRP itself is central to the greater virulence, whereas in hypothesis two, PRP identifies strains of greater virulence but is not itself the reason for the virtual monopoly exercised by type b strains in causing *H influenzae* meningitis. Whatever the result, we would wish to know, preferably at the molecular level, how PRP influences the occurrence of disease.

So in addition to the molecular genetics, it is essential to have a biologically relevant model of the infection, for microbial virulence is only a meaningful concept in the context of a host microbial relationship. We developed a meningitis model in which young rats were inoculated intranasally and developed bacteraemia and subsequently meningitis [10]. This model allowed a detailed study of the different stages in the pathogenesis: nasopharyngeal colonisation, invasion across the epithelial barrier of the respiratory tract, blood stream dissemination and localisation in the CNS following translocation of bacteria across the blood-meningeal barrier. The genes for capsule are chromosomal and occupy a relatively large region of 30–40 kb, about 1–2% of the entire bacterial genome. Although capsule is a surface polymer, the proteins that make it are located inside the cell and comprise the enzymes required to synthesise the precursor capsular polysaccharide, transport and polymerise it. Other genes assemble the polymer on the surface, altogether a complex process. Interestingly, these

Fig 4. *A gram stain of spinal fluid revealing numerous extracellular bacteria, gram-negative coccobaccillary organisms and inflammatory cells.* Note that virtually all the organisms are extracellular, an indication of their resistance to opsonophagocytosis.

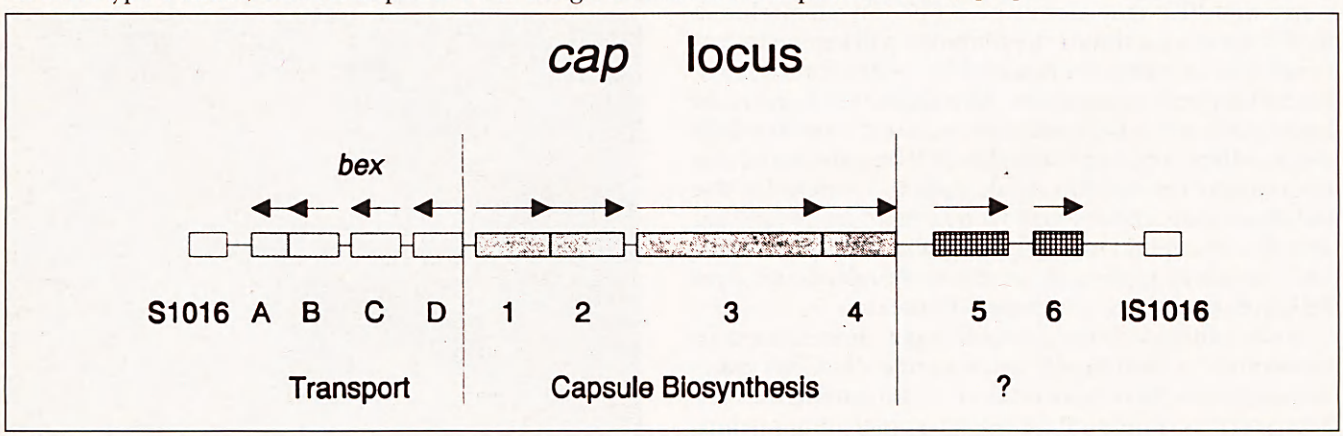


genes, with one notable exception, are present in two copies in most type b strains. Each of these duplicated regions comprises nine or ten genes—for as mentioned above, one repeat has an incomplete copy of one of the genes.

In the complete set, the genes are organised according to functions (Fig 5): for example, a set of four genes involved in export of capsule, a further set of four genes involved specifically in biosynthesis of PRP and a further set of two genes also involved in transport or assembly. Mutations in any one of these genes interfere with capsulation and allow us to analyse their specific function. In more general terms, they also provide us with the basic information needed to tackle Koch's postulates at the molecular level. By creating a genetically defined mutant, we can compare the behaviour of the wild-type and its isogenic capsule deficient mutant in our rat model and ask where in the sequence of the infection the capsule deficient mutant is defective. The mutant is able to colonise the nasopharynx but fails to cause bacteraemia or meningitis. In contrast, the wild-type causes bacteraemia and meningitis in a high proportion of inoculated rats. Further experiments showed that the effect of capsule was to promote the survival of the organisms in the blood. Interestingly, capsule down-regulates the efficiency with which organisms interact with host cells. Using human umbilical venous endothelial cells, we showed that attachment to endothelial cells is much greater for capsule deficient than for the encapsulated organisms [11], but once in the bloodstream the rare encapsulated strain can survive whereas the capsule deficient mutant cannot. However, capsule deficient strains are removed so efficiently once they translocate across epithelial and endothelial cells into the bloodstream that they cannot establish more than transient bacteraemia. These experiments shed some insight into the importance of capsule in promoting survival of *H influenzae* in the blood but also indicate that far from promoting attachment and translocation across cells, negatively charged polysaccharides down–regulate the efficiency with which encapsulated bacteria interact and invade epithelia and endothelia. This is just as well, for successful invasion must be a rare event if the bacterium is not to overwhelm too many of its hosts: such a strategy, taken to an extreme, would be counterproductive to its successful transmission and survival.

Does b capsule have properties denied to other capsular types of *H influenzae*? To approach this question, it was necessary to construct a series of isogenic strains, each expressing a different capsule but in the same background. This was achieved by transformation since capsular serotype specificity is a function of the central segment of *cap*. Now it became possible to compare the pathogenicity of the six different capsular serotypes in the animal model. The results were unequivocal and interesting, especially when bearing in mind clinical experience with encapsulated *H influenzae* infection. The type b variant was significantly more virulent [12]. Furthermore, there was an exquisite association between bacteraemia of a thousand or more organisms per ml and the probability of meningitis. Virtually all rats infected with the type b variant developed meningitis; none of those with the serotypes c, d or e developed bacteraemia or meningitis. These results parallel clinical experience since type b disease is the rule; much less frequent are instances of type a and type f bacteraemia, usually in not so healthy hosts; and the others are so rare that they are written up as case reports. Results indicate that PRP, the serotype b capsule, does indeed confer greater vir-

Fig 5. *The gene locus (cap) for type b capsule.* Chromosomal genes for the biosynthesis of the poly-ribosyl-ribotol capsular polysaccharide are arranged in three clusters. The bex genes (A, B, C, D, indicated by light grey polysaccharide from across the cytoplasm membrane. Four genes (1, 2, 3, 4, indicated by dark grey rectangles) are thought to be required for the biosynthesis of the polymer. Two genes (marked 5, 6) are of uncertain function. All 10 genes are flanked by the insertion element IS-1016 (white rectangles) so as to constitute a compound transposon. In most type b strain, there is duplication of the genes with the exception of bex A [17].



ulence upon *H influenzae* than do other capsular types. Furthermore, the quantitative differences between bacteraemia caused by the type b variant as compared to those making other capsules were such that meningitis occurred frequently with type b organisms and not at all with other capsular serotypes. This is not to say that b capsule is all there is to the pathogenic personality of *H influenzae*. Other factors are important: for instance, PRP itself does not cause any tissue injury. The inflammation in the meninges is caused by determinants encoded by other genes, notably those that are responsible for the elaboration of lipopolysaccharide, more commonly known as endotoxin and of which Lewis Thomas has written in his prescient way, keenly and graphically anticipating the era of research into cytokines [13].

> 'It is the information carried by bacteria that we cannot abide. For example they display lipopolysaccharide endotoxin in their walls, and these macromolecules are read by our tissues as the very worst of bad news. When we sense lipopolysaccharide, we are likely to turn on every defence at our disposal; we will bomb, defoliate, blockade, seal off, and destroy all the tissues in the area. Leukocytes become more actively phagocytic, release lysosomal enzymes, turn sticky, and aggregate together in dense masses, occluding capillaries and shutting off the blood supply. Complement is switched on at the right point in its sequence to release chemotactic signals, calling in leukocytes from everywhere. Vessels become hyper-reactive so that physiological concentrations of our own hormones suddenly possess necrotising properties. Pyrogen is released from leukocytes, adding fever to haemorrhage, necrosis, and shock'.

Man

Molecular genetic techniques have established the primacy of PRP as a virulence factor. Further studies on more than 2,000 carrier and disease strains, isolated from all over the world and over the past 40 years, have established that the genes for b capsule code for virtually identical proteins in these different organisms and that antigenic variation of type b epitopes is either exceedingly rare or nonexistent. The invariance of this surface structure among disease causing isolates is important when considering the capsule as a target for protective immune responses through serum antibodies. In the late sixties, in the USA, work began in earnest to develop a vaccine. Since PRP is a relatively simple, innocuous sugar and can safely be injected into animals or man, it was reasonable to consider whether PRP could be used as a vaccine in children. But PRP is a relatively poor immunogen in children under 18 months to two years old. Since 75% of invasive Hib disease, including meningitis, occurs in children less than two years, using PRP alone clearly is not the answer. The poor immune response to PRP is typical of so called thymus (T) cell independent antigens which do not prime for a T dependent response; also, the maturation of immunological competence in children, perhaps in the form of specialised antigen processing cells, is delayed until late in the second year.

To improve the response, use was made of the hapten-carrier principle, the subject of immunological research dating from the 1920s which showed that T cell independent antigens could be made T cell dependent by covalent coupling of the polysaccharide to protein [14]. Over the past several years the variables of the coupling process, the ratio of protein to carbohydrate, the nature of the protein carrier, and the size of the polysaccharide polymer have been studied in depth, in particular by Anderson and David Smith from the University of Rochester, New York, and Raquel Schneerson and John Robbins from the National Institutes of Health, Bethesda. Their results indicate that several PRP conjugates, now commercially available, can induce a protective immune response in infants as young as two months and that these conjugates are extremely safe.

Starting in 1984, the Oxford Hib Study Group set about the task of investigating the appropriateness of a routine immunisation programme against Hib. A prospective study was needed to establish the impact of the disease in the Oxford region as a guide to the problem in the UK as a whole. It showed that the problem of Hib disease had been seriously underestimated at the national level. The data indicated that about 1 in 600 children in the UK contract invasive Hib disease and about 70% of these life threatening infections are meningitis. This amounts to more than 1,300 cases each year, with more than 60 deaths. These figures, which resemble the deaths and disability caused by epidemic polio in the UK before the introduction of Sabin polio vaccine, made a compelling case for immunisation of infants. The next step was to test the safety and immunogenicity of Hib conjugates. In a cohort of 100 children, the Hib Study Group showed that conjugate PRP vaccine was well tolerated and that it was immunogenic when given to infants aged three, five and nine months. At this point, the Department of Health advised a change to the accelerated immunisation schedule of two, three and four months. No data existed anywhere in the world for children immunised as young as this and therefore a second immunogenicity and safety study was performed in Oxford. Again, the conjugate was well tolerated, there were no serious adverse effects, and the level of serum antibodies indicated that these extremely young infants made an excellent antibody response [15].

Could the vaccine be introduced as a routine within the National Health Service and would it prevent disease? To study this, infants were offered Hib conjugate vaccine in Oxford, Kettering, Northampton and Aylesbury and prospective case ascertainment of invasive Hib disease was carried out in the Oxford region among both immunised and non-immunised children;

12 cases occurred in children not offered the vaccine but none occurred in immunised children. This was a most encouraging result, realised literally days before the nation began routine immunisation of children with Hib conjugate vaccines [16]. Not the least of the important messages from the implementation study was that acceptance of the vaccine was extremely good; more than 90% of the children offered the vaccine were immunised.

Penelope waited 10 years for her Ulysses to return; the Hib Odyssey has been somewhat longer, a little more than 20 years, but an exciting adventure it has been. The routine immunisation programme in the UK is now on a secure basis and I believe that in a few years invasive Hib disease will be virtually eliminated from Europe and North America, and in the not too distant future, from many other countries as well.

Acknowledgements

It remains for me to acknowledge the contributions and hard work of the many people with whom I have had the good fortune to work, both in my laboratories in the USA and over the past eight years in Oxford. In particular, I wish to mention the sterling work of the Hib Study Group and thank all the health professionals in the Oxford Region for their dedication.

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17 Adapted from a figure drawn by J S Kroll. The figure summarises some unpublished data. The author wishes to acknowledge the contributions of J van Eldere, L Brophy and J Simon Kroll.

Address for correspondence: Professor E R Moxon, Department of Paediatrics, John Radcliffe Hospital, Oxford.