Pathogenicity and the microbe *in vivo*

The 1989 Fred Griffith Review Lecture

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When asked to give the Fred Griffith Review Lecture I was delighted for two reasons. First, it is an honour to provide one of the Society's named Lectures. Second, I was pleased that the Society had chosen someone interested in pathogenicity. The Griffith experiment is rightly renowned for heralding the demonstration of DNA as the primary genetic material. It was also a significant step in studies of pathogenicity.

In its new form, the Griffith Review Lecture should be reminiscent and personal. While preparing for it, I enjoyed looking back over 40 years' work on microbial pathogenicity. I have been fortunate to witness a great revival of interest in the subject that hooked me when I joined the Microbiological Research Establishment (MRE) at Porton, in 1947. At that time the subject was moribund. Now microbial pathogenicity is one of the most popular areas of microbiology. Many young people are attracted to it. The last Fleming Award was awarded to Gordon Dougan and a joint recipient of the next is Graham Boulnois: both are younger scientists involved with pathogenicity.

Two themes have dominated my research and writing. Pathogenicity is a multifactorial property. And, major attention should be given to the behaviour of the microbe *in vivo*. The action is there. We seek to explain infection and disease, and results gained *in vitro* must always be scrutinized with this in mind (Smith, 1958). The second theme is the subject of my lecture. I shall deal only with bacteria, although the principle applies to all types of microbes. Many of the examples come from my own research, as befits the Griffith Review Lecture. The last section describes recent work and contains more detail than the other sections.

The cardinal requirements for pathogenicity are abilities to grow in the host, to interfere with host defence

and to damage the host. Infection and penetration of mucous surfaces must also be added for the many microbes that invade over these surfaces (Smith, 1984). The goal of studies on pathogenicity is to identify the determinants of these requirements and to relate their chemical structure to biological function. Part of achieving this goal is to show that the determinant is either present or produced *in vivo*.

The environment *in vivo* influences bacterial pathogenicity by affecting growth and the production of virulence determinants. In studying both aspects, the relevant host factors should be identified and the bacterial reaction to them explored. The next section deals with these matters in relation to growth *in vivo* and the following one discusses them with respect to the formation of virulence determinants.

The influence of the environment *in vivo* on bacterial growth

Growth rate is important in pathogenicity. A fast-growing pathogen can overwhelm the initial, non-specific defences and cause disease before the more powerful immune defences can operate fully. A slower-growing one is more prone to both types of defence. Early measurements of the doubling times of *Salmonella typhimurium* and *Escherichia coli* in mice (Meynell & Subbaiah, 1963; Maw & Meynell, 1968; Polk & Miles, 1973) have led to the impression that multiplication of pathogens *in vivo* is slow, especially at the beginning of infection (Smith, 1984; Brown & Williams, 1985; Ruben, 1986). This has been interpreted as indicating that nutritional conditions *in vivo* may be limiting. Undoubtedly for some pathogens multiplication is slow. More recent determinations of growth rates *in vivo* suggest, however, that slow multiplication rates and limiting growth conditions are by no means universal.

Abbreviations: CMP-NANA, cytidine 5'-monophospho-N-acetylneuraminic acid; LPS, lipopolysaccharide; mAb, monoclonal antibody; OMP, outer-membrane protein; PMN, polymorphonuclear; RIF, resistance-inducing factor.

Measurement of multiplication rate *in vivo*

There are no well-proven, universally applicable methods for measuring doubling times of bacterial pathogens in tissues. Usually, increases in bacterial populations of the blood, lymph nodes, spleen or liver are recorded at long intervals (O'Callaghan *et al.*, 1988; Curtiss *et al.*, 1988) but sometimes more frequently (Keppie *et al.*, 1955; Ruben, 1986). They are the resultants of bacterial multiplication, and destruction or removal by the host (Smith, 1976; Ruben, 1986). If a population increases very rapidly there is no doubt that doubling times are short, dominating the overall numbers despite any effect of host defences. When the population increases slowly or even decreases (Polk & Miles, 1973) multiplication rates are not clear.

The first attempts to measure true doubling times *in vivo* relied on a genetic marker distributing to only one of the two daughter cells in each succeeding generation (Meynell & Subbaiah, 1963; Maw & Meynell, 1968; Polk & Miles, 1973). The proportion of the bacterial population carrying non-replicating markers, examined at intervals during infection, revealed the number of ensuing generations. The marker was assumed not to affect either multiplication or death of the organism. The genetic manipulation required restricted the method to *E. coli* and salmonellae. In the spleens of mice, *S. typhimurium* doubled every 5 h for 4 d after intravenous inoculation; and every 6 h for 24 h after direct inoculation into the intestine. Later, Hormaeche (1980) calculated doubling times for *S. typhimurium* in the spleens of susceptible and resistant mice as 3 and 5 h respectively from 12-hourly observations over 2 d. In mouse muscle, *E. coli* failed to multiply for 4 h after inoculation and doubled only twice in the next 3 h (Polk & Miles, 1973). These figures contrasted with doubling times of 20–30 min in laboratory media.

Doubling times have been calculated from the progressive loss of specific activity of bacterial populations harvested from animals at intervals after injecting radiolabelled pathogens. This method does not distinguish between dead and live organisms, and radiolabel can leach from both (Freter & O'Brien, 1981; Hooke *et al.*, 1985). For 7 h after injection into the kidneys of mice, *E. coli* and *Pseudomonas aeruginosa* appeared to double every 0.9 and 2.3 h respectively (Eudy & Burroughs, 1973). After inoculation of *Vibrio cholerae* into the upper bowel of infant mice, doubling times of about 2.5 h (Baselski *et al.*, 1978) and about 1 h (Sigel *et al.*, 1981) were indicated. These figures are open to question but they indicate higher multiplication rates than those quoted above.

More recently, the increase in ratio of wild-type organisms (which multiply *in vivo*) to those of tempera-

ture-sensitive mutants (which should not multiply *in vivo*), have been used to calculate doubling times. Tests are done to indicate that mutants and wild-types have the same susceptibility to host defences. Relative counts made at short intervals for 180 min after intraperitoneal inoculation of mice indicated doubling times of 33 min for *E. coli* and 20 min for *P. aeruginosa* (Hooke *et al.*, 1985). Similar experiments with *P. aeruginosa* in mouse lungs indicated a doubling time of 30 min over the first 5 h of infection (Sordelli *et al.*, 1985). These doubling times at the beginning of infection *in vivo* are similar to those seen *in vitro*. The method has potential for general use.

Even when slow multiplication *in vivo* is observed, this does not necessarily mean that limiting nutritional conditions exist. Host defences may restrict growth rate as well as remove or destroy the pathogen. In the muscle of living mice, little multiplication of *E. coli* occurred for 7 h after inoculation (Polk & Miles, 1973). The nutritional completeness of the muscle was indicated by inoculating recently killed mice. Multiplication occurred immediately and there were 2 to 3 generations in 4 h and 7 to 8 in 7 h. Similarly, doubling times of *S. typhimurium* in the intestines of mice were increased by removing the bacteriostatic commensals with streptomycin (Meynell & Subbaiah, 1963).

Nutrition *in vivo*: lack of knowledge

The crucial nutrients or environmental factors which determine the growth rate for particular pathogens *in vivo* are largely unknown, and unstudied. There are, however, a few exceptions. The influence of oxygen tension on the multiplication of various pathogens has been studied, e.g. the war-wound anaerobes (Wilson & Miles, 1946; Woods & Foster, 1964), abdominal abscess anaerobes (Onderdonk *et al.*, 1976) and staphylococci in the udder (Bassalik-Chabielska *et al.*, 1985). Growth of *Bacillus anthracis* in the blood of terminally ill guinea-pigs was inhibited by the antimetabolites 8-azaguanine, 8-azaxanthine, ethionine, α -amino-n-butyric acid and *p*-fluorophenylalanine. Annulment of these inhibitions by mixture with appropriate metabolites indicated that hypoxanthine, adenine, methionine, alanine, phenylalanine and tryptophan might aid growth *in vivo* (Tempest & Smith, 1957).

The most notable exception to the lack of knowledge is the erudite work on iron limitation. It began in the mid-1940s (Schade & Caroline, 1944, 1946), mushroomed in the 1970s and is still flourishing. There is no point in repeating good reviews (Weinberg, 1978; Bullen, 1981; Brown & Williams, 1985; Griffiths *et al.*, 1988). The work is an excellent example of the value of integrating studies *in vitro* with those *in vivo*. Iron was needed for

growth of pathogens *in vitro* when egg-white or serum was included in the media. Iron enhanced the virulence of many bacterial pathogens in various animal models. Chelation by transferrin and lactoferrin was shown to be responsible for scarcity of iron *in vivo*. Experiments *in vitro* under iron-limiting conditions, often in chemostats, demonstrated the production of siderophores, new tRNAs and hitherto unknown outer-membrane proteins (OMPs). Finally, all the products of iron limitation *in vitro* were found *in vivo*.

Nutrition and tissue localization: erythritol and brucellae

In the 1960s I became interested in another influence of nutrition on pathogens *in vivo*. A preferred nutrient localized in a certain site can be significant in concentrating infection there rather than in other tissues. *Corynebacterium renale* causes a severe kidney infection in cattle. When grown in bovine urine and urea-enriched peptone water it produces a urease and uses urea for growth (Lovell & Harvey, 1950). *Proteus mirabilis* causes severe damage to the kidneys of man and rats (Braude & Sieminski, 1960). Intracellular growth of *P. mirabilis* in tissue cultures of rat kidney epithelium was stimulated by urea and the optimum concentration (0.2%) was that found in kidney homogenates (Braude & Sieminski, 1960). Hence, growth stimulation by urea appears to contribute to these kidney localizations in addition to depression of non-specific defence mechanisms by salt concentration and ammonia (Pearce & Lowrie, 1972). Urease-less mutants of *C. renale* and *P. mirabilis* retained the renal predilection of their parent strains but growth in the medulla was more limited, renal damage less and recovery from infection more rapid (Lister, 1957; MacLaren, 1969).

My group at MRE investigated the tissue specificity of the brucellae (Smith *et al.*, 1961, 1962, 1965; Pearce *et al.*, 1962; Williams *et al.*, 1962, 1964; Anderson & Smith, 1965; Keppie *et al.*, 1965, 1967). Brucellosis is mainly a disease of cattle (caused chiefly by *Brucella abortus*), sheep and goats (*Brucella melitensis*) and pigs (*Brucella suis*). In pregnant animals brucellae proliferate extensively in the placenta, the foetal fluids and the chorions, leading to abortion. In nonpregnant animals, brucellae do not grow abnormally and have no marked affinity for particular tissues. In the male there is a localization in the genitalia. Human brucellosis is an undulating fever, often chronic, with no overwhelming growth of brucellae or marked localization; abortion is rare. This is also the situation for brucellosis of guinea-pigs, rats and rabbits.

Pregnant cows, infected with *B. abortus*, were killed when they were about to abort. A survey of their organs for brucellae showed a total of about 1×10^{13} and over 90% in the foetal placenta, chorion and foetal fluids.

Extracts of these tissues were the most active when all foetal and maternal tissues were surveyed for a growth stimulant of *B. abortus*. The stimulant was identified as erythritol and chemical analysis showed it to be concentrated in the tissues which become heavily infected. Erythritol is a preferred nutrient for *B. abortus*. When the latter was growing in a medium containing glucose, and 1/1000 times less ^{14}C -labelled erythritol was added, erythritol was used instead of glucose and the ^{14}C was found in all components of the brucellae. It enhanced *B. abortus* infection in new-born calves and erythritol analogues inhibited the growth of *B. abortus in vitro* and *in vivo*. Most satisfactorily, erythritol inhibited the growth of *B. abortus* strain S19, which had been used for years as a safe vaccine in the field without producing abortions.

Turning to brucellosis in other species, erythritol was found in the placenta of goats, sheep and pigs and stimulated the growth of *B. melitensis* and *B. suis in vitro* and *in vivo*. It was not found in the placenta of humans, guinea-pigs, rats and rabbits. Furthermore, testes and seminal vesicles of the domestic species contained erythritol, but not the seminal vesicles of man.

In summary, the presence of erythritol, a growth stimulant for brucellae, explained the localization of infection in cattle, goats, sheep and pigs and why brucella infections in other species such as man do not show such tissue predilection.

Future studies on nutrition and metabolism

Despite the success of research on iron limitation and of that on tissue localization, nutrition and metabolism *in vivo* remains the most understudied area of bacterial pathogenicity. There is a great gap in knowledge. The situation is in stark contrast to that on viral pathogenicity, where replication is the area of choice for basic studies (Smith, 1989). There is a pressing need for reliable methods to measure growth rate *in vivo* that can be applied to a wide range of pathogens. The use of temperature-sensitive mutants (see above) has possibilities here.

Bacterial nutrients other than iron, e.g. PO_4^{3-} , Zn^{2+} and Mg^{2+} , may also be limiting in some tissues or some hosts (Brown & Williams, 1985). Possible limitations should be investigated by growth experiments with avirulent or attenuated strains (virulent strains may cope with the deficiency), in serum-containing media, with and without supplementation by the suspect material. The nutrients essential for normal growth *in vivo* and those which may stimulate rapid growth later in infection could also be identified by appropriate modifications of the system. Once identified, their uptake and metabolism could be studied by *in vitro* and *in vivo*

methods similar to those used for iron limitation. The possible implication of preferred nutrients in tissue localizations, e.g. *Listeria monocytogenes* in the brain, should be kept in mind. Preliminary investigations on growth stimulation by tissue extracts should indicate whether studies should be continued as for the brucellae.

Nutritionally deficient mutants may be useful. Such mutants of *S. typhimurium* are being prepared as vehicles for genetically engineered vaccines (Hoiseth & Stocker, 1981; Curtiss *et al.*, 1988; O'Callaghan *et al.*, 1988; Dougan, 1989). They cannot accomplish key biochemical reactions in the biosynthesis of certain aromatic amino acids and/or purines. Their mouse virulence is low, probably arising from impaired growth and multiplication *in vivo*, but this is not certain. The mutants show smaller increases in number than does the wild-type in bacterial contents of Peyer's patches, mesenteric lymph nodes, liver or spleen taken from animals killed at daily intervals. However, doubling times were not measured, so greater susceptibility to host defences may have contributed to the lower total populations. If mutants of this type were injected and their true multiplication rates were measured over short time periods *in vivo*, absent and available nutrients might be revealed by observing which mutants grew and which did not. Infection would be best followed in localized sites such as the peritoneal cavity, subcutaneous lesions or tissue chambers. Once revealed, the influence of the available nutrients on growth could be studied, e.g. by observing the effect of adding more. Although not strictly *in vivo*, recent nutritional studies on growth of *Legionella pneumophila* in human monocytes using tryptophan and thymidine auxotrophs are a step in the right direction (Mintz *et al.*, 1988). Mutants that are unable to synthesize many common metabolites and even higher M_r materials such as particular peptides or oligosaccharides (Salyers, 1989) could be prepared. The information gained might lead to metabolic studies using radiolabelled materials, not only *in vitro* but also *in vivo*, now that small quantities of *in vivo*-grown organisms can be harvested and examined directly by SDS-PAGE (see later).

To sum up, the nutritional and metabolic background to growth of pathogenic bacteria *in vivo* is under-studied. There is much to do and there are ways forward. Experts in microbial metabolism are needed in the field of pathogenicity.

The influence of the environment *in vivo* on virulence determinants

Behaviour is determined by genetic make-up and environment. The environment for bacterial pathogens during infection is complex and constantly changing. It

is different from that used *in vitro* for most experiments on pathogenicity. Hence, when pathogens are moved from one type of environment to the other, selection and phenotypic change occur. There are many examples. Gonococci provide some of the best. When *in vitro*-grown gonococci were inoculated into human volunteers, variants were selected which expressed pilins and OMPs of class II different from those formed by the inoculum (Swanson *et al.*, 1987, 1988). Growth of gonococci in subcutaneous guinea-pig chambers led to selection of a strain from a laboratory-grown culture which, unlike the latter, was relatively resistant to killing by human phagocytes, as are gonococci in urethral exudates (Penn *et al.*, 1977; Parsons *et al.*, 1985). Also, gonococci from these chambers and from urethral exudates are resistant to killing by fresh human serum (Penn *et al.*, 1977; Ward *et al.*, 1970). This resistance is, however, not due to selection of a strain but to induction by a host factor *in vivo*; it is lost phenotypically on culture *in vitro* in the absence of the host factor (Rittenberg *et al.*, 1977; Parsons *et al.*, 1985).

The changes that can occur when bacteria are transferred between *in vivo* and *in vitro* conditions have two implications for studies on pathogenicity. First, putative determinants of pathogenicity, indicated by experiments using *in vitro*-grown organisms, may not be produced *in vivo*. Second, and perhaps more important, virulence determinants found *in vivo* may be missed because they are not formed under arbitrarily chosen growth conditions *in vitro*. Nor is the consequence of such environmental change confined to determinants of pathogenicity. Killed vaccines prepared from *in vitro*-grown organisms or their products may be incomplete as regards immunizing antigens which are produced *in vivo* either by infection or by live attenuated vaccines (Smith, 1964; Brown *et al.*, 1988). Also, drugs adequate in tests against *in vitro*-grown bacteria may not be effective against pathogens *in vivo* (Smith, 1964, 1985; Brown & Williams, 1985). The message is obvious. Check on bacteria grown *in vivo*.

History of studies on bacteria grown *in vivo*

Despite the inescapable logic of the previous section, examination of bacteria grown *in vivo* has had a chequered history. About 1900, after the discovery of tetanus and diphtheria toxins in culture filtrates, Bail (Wilson & Miles, 1946) showed that sterile exudates from animals with anthrax, cholera, typhoid or plague promoted experimental infections. He coined the word 'aggressins' to describe bacterial products that are not necessarily toxic but keep defence mechanisms at bay, so allowing the pathogen to proliferate in the host. In this he was right, a big step in studies of pathogenicity. He was

wrong, however, in insisting that aggressins were only produced *in vivo*. Many aggressins were soon produced *in vitro* and shown to interfere with serum killing and ingestion by phagocytes (Wilson & Miles, 1946). Interest in bacteria grown *in vivo* then lapsed. There was much to be done with organisms grown *in vitro*. Little occurred until the 1950s, although some people (Dubos, 1954) kept alive the idea that bacteria *in vivo* could be different from those *in vitro*. Then, De & Chatterje (1953) showed that live *V. cholerae* caused fluid accumulation in ligated rabbit loops, the observation *in vivo* that heralded the recognition of cholera enterotoxin (Finkelstein & La Spalluto, 1970). And, there was some work on anthrax.

Soon after joining MRE in 1947, I suggested to the director, Dr D. W. Henderson (later to become an FRS and President of this Society) that to learn more about bacterial pathogenicity we should examine organisms grown *in vivo*. He backed the idea and advised work on anthrax. Also, no doubt thinking that an organic chemist might be unsafe on such a disease, he coupled me to a veterinary microbiologist, the late Dr James Keppie. It was a synergistic relationship. Anthrax kills many animal species and usually there is a massive terminal bacteraemia. It was the vehicle for proof of Koch's Postulates in 1876. Not long afterwards, tetanus and diphtheria toxins were produced in culture filtrates; so it is not surprising that, from the earliest times, attempts were made to demonstrate an anthrax toxin in laboratory cultures. All were without avail and the cause of death from anthrax remained an enigma for many years. Henderson's suggestion of using anthrax for studying organisms grown *in vivo* carried two advantages. First, the large number of bacteria in the blood would help in establishing a method for separating organisms from infected animals. Second, there was a major problem in pathogenicity to solve by the use of such organisms or their products.

Separation and properties of in vivo-grown anthrax bacilli. Large guinea-pigs (800–1000 g) were infected intraperitoneally and intrapulmonarily with virulent *B. anthracis*. At death, the thoracic and peritoneal exudates were collected together with blood [containing about 1×10^9 chains (2–8 bacilli) per ml] that exuded into the thoracic cavity, after cutting out the heart and lungs. Differential centrifugation of the mixed material at 4 °C separated bacteria from blood cells and body fluids. About 1.5–2.0 g dry *B. anthracis* was obtained from 100 guinea-pigs with contaminating blood cell substance less than 1% (Smith *et al.*, 1953a).

The *in vivo*-grown anthrax bacilli showed profound differences in membrane and wall characteristics compared with the same strain grown *in vitro*. After centrifugation in saline and suspension in water, they became so swollen that they could no longer be deposited

by a similar centrifugation; this swelling did not occur for organisms grown *in vitro* on four different media and harvested at four different times (i.e. 16 different samples). Also, addition of ammonium carbonate (0.16%, w/v) to the aqueous suspension lysed the *in vivo*-grown organisms completely but had no effect on the *in vitro*-grown bacteria (Smith *et al.*, 1953b). In addition to these membrane and wall differences, the *in vivo*-grown organisms were more capsulated and more resistant to phagocytosis than the *in vitro*-grown organisms (Smith *et al.*, 1953b).

The method of separating *in vivo*-grown organisms from infected animals was shown to work for *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *L. monocytogenes* (Smith *et al.*, 1953a). Later, it was used with some modifications for separating large quantities of *Yersinia pestis* from infected guinea-pigs (Smith *et al.*, 1960) and of *B. abortus* from foetal tissues of infected pregnant cows (Smith *et al.*, 1961).

The discovery of the anthrax toxin. When Keppie and I started work, most speculations on the cause of death in anthrax were based on possible effects of the massive terminal bacteraemia, e.g. blockage of the capillaries or usage of oxygen. The first step, therefore, was to see whether or not the large bacterial count in the blood was necessary for death. During the last 12 h before death of guinea-pigs from anthrax, the number of bacterial chains rises from about 3×10^5 to 1×10^9 ml⁻¹, and there is a constant relationship between the blood count and time to death. This made possible the treatment of guinea-pigs with high doses of streptomycin at known stages in the final bacteraemia. The bacilli stopped multiplying in 1 h and the blood was completely cleared by 6 h. The treatment saved guinea-pigs provided it was given 8 h or more before they would have died and when their blood count was about 3×10^6 . Thereafter, the treatment was unsuccessful and the guinea-pigs died *despite the absence of anthrax bacilli in the blood*. The coup-de-grace had been given when the count was about 1×10^6 ml⁻¹, the critical point in the bacteraemia. This experiment (Keppie *et al.*, 1955) disposed of many previous theories for death and suggested that a toxin might be responsible.

The nature of a possible toxin was indicated by pathophysiological studies on guinea-pigs dying of anthrax. These indicated oligoemic shock caused by loss of fluid from the blood (Smith *et al.*, 1954, 1955a). When bacteria-free plasma from guinea-pigs dying of anthrax was injected subcutaneously into guinea-pigs, an area of oedema formed over the next 18 h. This effect was neutralized by antiserum prepared against live anthrax organisms. Larger quantities of the plasma killed both mice and guinea-pigs, with pathological effects similar to those seen in infection; and, again, the lethal effect was neutralized by anthrax antiserum. Thus, after 60 years of

fruitless research *in vitro*, the anthrax toxin had been recognized by looking *in vivo* (Smith & Keppie, 1954; Smith *et al.*, 1955b). It was some time before people were convinced. The toxin was produced *in vitro* (Harris-Smith *et al.*, 1958) and shown to have three components (Smith & Stoner, 1967); little did we know at the time that other toxins would also prove to be multicomponent.

Subsequent studies with other bacteria grown in vivo. *In vivo*-grown tubercle bacilli were also examined in the 1950s. Segal & Bloch (1956, 1957) separated them from infected mouse lungs. They were more virulent than *in vitro*-grown organisms and had different surface, metabolic and chemical properties. Later work uncovered further differences (Barksdale & Kim, 1977).

Despite these demonstrations in the 1950s that bacteria could be harvested from infected animals and that examining them could be rewarding, the idea did not catch on. Pathogenicity was not a popular area of microbiology. Separating *in vivo*-grown organisms in quantity was inconvenient and often dangerous. Not everyone had the facilities of MRE for animal experiments. There was some spasmodic effort. *Y. pestis*, *Pasteurella multocida*, *Streptococcus pyogenes* and *Staphylococcus aureus* harvested from infected animals were shown to be more virulent than organisms grown *in vitro* and different chemically, metabolically and antigenically (Smith, 1964). At MRE we harvested *Y. pestis* from infected guinea-pigs to investigate a paradox regarding its toxin. Although guinea-pigs and mice were both killed by *Y. pestis*, a toxin that had been isolated from *in vitro*-grown organisms killed only mice. A guinea-pig toxin was recognized when extracts of *Y. pestis* grown *in vivo* killed both guinea-pigs and mice (Cocking *et al.*, 1960): it had two components (Stanley & Smith, 1967). Also, we investigated the ability of *B. abortus* to survive and grow within phagocytes, using organisms harvested from foetal placentae of infected cows. They resisted killing by bovine phagocytes better than *in vitro*-grown organisms (Smith & FitzGeorge, 1964). This observation led to the recognition of a cell wall component which interfered with intracellular killing (Frost *et al.*, 1972).

It was the 1980s before studies of *in vivo*-grown organisms really took off. By then, pathogenicity was popular and techniques had become available for analysing the surface components of small numbers of bacteria that could be harvested from animals. Now, examining the characteristics of bacteria grown *in vivo* is a vogue subject.

Present studies

There are two arms to the present effort. First, evidence is sought for the production *in vivo* of putative virulence

determinants that have been indicated by experiments *in vitro*. Second, *in vivo*- and *in vitro*-grown bacteria are examined for biological and chemical differences which may indicate hitherto unknown virulence determinants.

Demonstrating extracellular toxins *in vivo* has not been particularly difficult. Early in microbiology, the effects of diphtheria and tetanus toxins were seen to be identical with those of disease (Wilson & Miles, 1946; van Heyningen, 1955), and such comparisons have continued over the years for many other toxins, including the various enterotoxins (Stephen & Pietrowski, 1986). In many cases the evidence has been increased by passive or active immunoprotection of man and animals against the relevant diseases with antisera or toxoids (Stephen & Pietrowski, 1986). Also, toxins and their antibodies have been detected in tissues or blood by serological methods.

Demonstrating cellular virulence determinants was a problem but this has now yielded to modern techniques. First, monospecific, fluorescently labelled antibodies are used to demonstrate virulence determinants on the surfaces of bacteria in tissue sections. Second, SDS-PAGE allows meaningful examinations of unfractionated components in the relatively small numbers of bacteria that can be obtained from *in vivo* sources. Gel profiles of OMPs, lipopolysaccharides (LPS) and other components can be compared with those from *in vitro*-grown organisms. They can then be immunoblotted with polyclonal antisera or monoclonal antibodies that recognize specific determinants. Patients' sera can be examined for antibodies to the relevant determinant.

Table 1 lists examples of putative virulence determinants that have been demonstrated *in vivo*. Iron-regulated OMPs predominate. Some are virulence determinants since they aid growth *in vivo* by acting as vehicles for iron transport (Griffiths *et al.*, 1988), but any role for the others has yet to be proved. Some putative determinants of other aspects of pathogenicity are recorded in Table 1, e.g. the antiphagocytic capsular polysaccharide of staphylococci (Karakawa *et al.*, 1988).

Table 2 lists recent demonstrations of differences between *in vivo*- and *in vitro*-grown organisms which might lead to recognition of new determinants of pathogenicity. Two of the observations on gonococci have been followed up. Resistance to complement-mediated killing by serum is dealt with later. Studies on the increased resistance to killing by phagocytes are summarized below.

Whether or not gonococci survive within human phagocytes has been much discussed (Parsons *et al.*, 1985; Rest & Shafer, 1989). Old laboratory strains grown *in vitro* are largely killed. A few organisms, however, survive intracellularly (Parsons *et al.*, 1985; Rest & Shafer, 1989). And, there is evidence (Parsons *et al.*, 1985, 1986), now gaining acceptance (Rest & Shafer,

Table 1. Confirmation that putative surface virulence determinants detected *in vitro* are produced *in vivo*

Bacterial species	Host	Site of infection	Putative determinant demonstrated <i>in vivo</i> (and/or its antibody)	Reference
<i>Pseudomonas aeruginosa</i>	Man	Lung, cystic fibrosis	Iron-regulated OMPs	Anwar <i>et al.</i> (1984)
			LPS with O polysaccharide	Brown <i>et al.</i> (1984)
	Rat	Burn wound	Iron-regulated OMPs	Cochrane <i>et al.</i> (1988b)
<i>Escherichia coli</i>	Man	Lung	Iron-regulated OMPs	Ward <i>et al.</i> (1988)
		Urine	Iron-regulated OMPs	Cochrane <i>et al.</i> (1988a)
	Guinea-pig	Peritoneal cavity	Enterochelin	Lam <i>et al.</i> (1984)
<i>Proteus mirabilis</i>	Man	Urine	Iron-regulated OMPs	Griffiths <i>et al.</i> (1985)
			Enterochelin	Moore & Earhart (1981)
<i>Klebsiella pneumoniae</i>	Man	Urine	Iron-regulated OMPs	Griffiths <i>et al.</i> (1983)
			Enterochelin	
<i>Vibrio cholerae</i>	Infant rabbit	Intestine	Iron-regulated OMPs*	Lam <i>et al.</i> (1984)
		Intestine	Iron-regulated OMPs	Shand <i>et al.</i> (1985)
<i>Shigella flexneri</i>	Man and monkey	Intestine	Iron-regulated OMPs	Lam <i>et al.</i> (1984)
		Intestine	Iron-regulated OMPs	Shand <i>et al.</i> (1985)
<i>Streptococcus pyogenes</i>	Mouse	Implanted chambers	Iron-regulated OMPs*	Kadurugamuwa <i>et al.</i> (1988)
			Iron-regulated OMPs	Sciortino <i>et al.</i> (1983)
<i>Staphylococcus aureus</i>	Rat	Blood	'Invasion' plasmid proteins b and c	Hale & Formal (1988)
		Blood	'Invasion' plasmid proteins b and c	Cleary <i>et al.</i> (1989)
Group B streptococci	Guinea-pig	Skin	Surface antigens (and O and S lysins)	Duncan (1983)
			Capsular polysaccharide	Arbeit & Nelles (1987)
Group B streptococci	Rabbit	Implanted chambers	Capsular polysaccharide	Arbeit & Dunn (1987)
			Capsular polysaccharide	Wagner <i>et al.</i> (1982)

* Repressed by injecting iron into the cavity.

1989), that conditions *in vivo* select gonococci which survive and grow intracellularly. This was indicated by electron microscopy and phagocytosis tests on gonococci in urethral exudates (Casey *et al.*, 1980) and by phagocytosis tests on gonococci selected by growth in guinea-pig subcutaneous chambers (Penn *et al.*, 1977; Parsons *et al.*, 1985, 1986). Surface washes of the latter, but not those of the parent laboratory strain, neutralized the ability of antigenococcal serum to abolish intracellular survival of the *in vivo*-selected gonococci. SDS-PAGE of their surface washes revealed a 20 kDa component not present in washes of the laboratory strain. The component, purified to form a single band on SDS-PAGE, was a lipoprotein containing much glutamic acid. When the laboratory strain was pretreated with the lipoprotein, it gained resistance to killing by human phagocytes. Also, intracellular survival of the strain selected *in vivo* was abolished by monospecific mouse antiserum against the purified lipoprotein. The latter appears, therefore, to be a determinant of intracellular survival of gonococci

(Parsons *et al.*, 1986) which contributes to overall pathogenicity (Parsons *et al.*, 1985).

Bacteria grown *in vitro* can have properties related to virulence which are either not found *in vivo* or are much reduced (Table 2). Serum resistance of some *E. coli* strains was reduced by growth *in vivo* (Finn *et al.*, 1982). *Bacteroides fragilis* grown in mouse peritoneal chambers rapidly lost its large capsule and resistance to phagocytosis but retained a small capsule (Patrick, 1988). This is interesting because *B. fragilis*, selected by growing in an abscess model *in vivo* and minimally subcultured, showed increased capsulation compared with laboratory strains (Simon *et al.*, 1982). The role in pathogenicity and human infection of the small capsules should be investigated. A result similar to that for *B. fragilis* is the increased susceptibility of *in vivo*-grown *P. aeruginosa* to phagocytosis compared with *in vitro*-grown organisms (Kelly *et al.*, 1987). Obviously, virulence attributes detected by study of *in vitro*-grown organisms must not be assumed always to be active *in vivo*.

Table 2. *Properties of in vivo-grown bacteria different from those of in vitro-grown organisms and related to pathogenicity*

Bacterial species	Host	Source of bacteria	Property of <i>in vivo</i> organisms different from <i>in vitro</i> organisms	Reference
<i>Escherichia coli</i>	Mouse	Implanted chambers	Serum sensitivity	Finn <i>et al.</i> (1982)
	Rabbit		Membrane proteins	
	Mouse	Peritoneal cavity	Fimbrial variation	Nowicki <i>et al.</i> (1986)
	Man	Urine	Pilus variation	Kisielius <i>et al.</i> (1989)
<i>Bacteroides fragilis</i>	Mouse	Implanted chambers	Less capsule	Patrick (1988)
<i>Staphylococcus aureus</i>	Sheep	Implanted dialysis tubes	More virulent Phagocytosis resistance Extra antigen	Watson (1983)
<i>Neisseria gonorrhoeae</i>	Man	Urethral exudate	Serum resistance	Ward <i>et al.</i> (1970)
			Resistance to phagocyte killing	Casey <i>et al.</i> (1980)
			Pilins	Swanson <i>et al.</i> (1987)
	Guinea-pig	Implanted chambers	Protein IIs	Swanson <i>et al.</i> (1988)
			LPS components	Mandrell <i>et al.</i> (1990)
			Serum resistance	Parsons <i>et al.</i> (1985)
			Phagocyte killing	
<i>Haemophilus influenzae</i>	Rat	Blood	Serum resistance	Kuratana <i>et al.</i> (1989)
<i>Pseudomonas aeruginosa</i>	Mouse	Implanted chambers	Susceptible to phagocytosis	Kelly <i>et al.</i> (1987)
	Mouse	Implanted chambers		
	Rat	Implanted chambers	LPS components	Kelly <i>et al.</i> (1989)
<i>Vibrio cholerae</i>	Rabbit	Intestine	Surface antigens (7-8)	Jonson <i>et al.</i> (1989)
<i>Campylobacter fetus</i>	Man	Intestine	Surface layer Phagocytosis and serum resistance	Fujimoto <i>et al.</i> (1989)
<i>Campylobacter coli</i>	Rabbit	Intestine	Flagellar variation	Logan <i>et al.</i> (1989)

Future studies: specification of host factors and corresponding virulence determinants

Investigations on *in vivo*-grown bacteria will continue and expand along the two lines described in the previous section. There should, however, be a further development: identification of particular host factors which cause the production *in vivo* of defined virulence determinants.

Two attitudes exist in most present studies. First, the influence of the undefined environment *in vivo* is accepted as a whole when bacteria are grown in experimental animals or separated from patients for examination of putative or new virulence determinants. Second, the effect, on production of virulence determinants, of environmental factors that might operate *in vivo* is studied *in vitro* without demonstrating that these factors do in fact operate *in vivo* (Brown & Williams, 1985; Miller *et al.*, 1989). Examples are as follows.

Most efforts to investigate possible host influences

stem from original indications that growth conditions *in vivo* may be limiting (see above). Early chemostat studies on both Gram-negative and Gram-positive organisms showed that limitation of Mg^{2+} , PO_4^{3-} , K^+ , NH_4^+ , glucose and other substrates which occur *in vivo* affected production of putative virulence determinants (Ellwood & Tempest, 1972; Ellwood, 1974). Later work tended to concentrate on the effect of iron limitation, but significant attention has been given to C, Mg^{2+} and PO_4^{3-} limitation (Brown & Williams, 1985). Much less effort has been directed to the effect of materials which might not be limiting *in vivo* but are required to produce virulence determinants, namely amino acids, peptides, sugars, purines, pyrimidines, nucleotides and even more complex compounds. The recent recognition that, sometimes, conditions may not be growth limiting *in vivo* may direct more attention to non-limiting substrates.

Demonstration that some pathogens regulate production of different virulence determinants by common mechanisms which are influenced by environmental factors (Betley *et al.*, 1986; Miller *et al.*, 1989; Finlay &

Falkow, 1989) is not surprising. Global regulatory networks in which a given environmental signal causes the coordinate induction or repression of diverse and unlinked genes is well known (Gottesman, 1984) and appears to involve DNA supercoiling and protein phosphorylation (Dorman *et al.*, 1988; Bhriain *et al.*, 1989; Bourret *et al.*, 1989). However, the recent focus on virulence determinants has emphasized the importance of environmental factors in influencing their production. Osmolarity, pH, temperature and certain amino acids affected the action of the Tox-R gene of *V. cholerae*, which regulates the formation of enterotoxin, pili and other products (Betley *et al.*, 1986). Virulence factors of *Bordetella pertussis* are either not formed or decreased by lowering the temperature from 37 °C to 25 °C and by raising the levels of MgSO₄ and nicotinic acid (Miller *et al.*, 1989). *Yersinia* spp. cease to express several determinants of pathogenicity at 25 °C compared with 37 °C or when Ca²⁺ levels are raised (Finlay & Falkow, 1989). Grown at 30 °C, *Shigella* spp. lose virulence and ability to invade cells (Miller *et al.*, 1989). A neuraminidase- and trypsin-labile material on the surfaces of epithelial cells appears, on contact with *Salmonella* spp., to trigger *de novo* synthesis of bacterial proteins required for adherence and invasion (Finlay & Falkow, 1989). Bicarbonate levels affect the production of at least one component of the anthrax toxin (Bartkus & Leppla, 1989). Production of toxic shock syndrome toxin is affected by Mg²⁺ concentration (Kass *et al.*, 1988). All these factors could switch on or off production of virulence factors *in vivo*, but the only one known to operate is temperature. *V. cholerae*, *B. pertussis*, *Yersinia* spp. and *Shigella* spp. express their virulence attributes at mammalian body temperatures and not at those of vectors and external habitats (Finlay & Falkow, 1989; Miller *et al.*, 1989). The difficulty of proving whether the other environmental factors influence behaviour *in vivo* has been stressed (Miller *et al.*, 1989).

We must persist in attempts to identify substrates present *in vivo* that are used for production of defined virulence determinants and, also, the host factors that control regulation of production. This has been done for iron limitation, which clearly occurs *in vivo* and affects many pathogens. It results in production *in vivo* of siderophores and OMPs which facilitate iron uptake and are, therefore, determinants of one requirement of pathogenicity – multiplication. Some toxins are also induced by low iron concentrations, e.g. diphtheria toxin, shiga toxin and exotoxin A of *P. aeruginosa* (Miller *et al.*, 1989). Turning to non-limiting conditions, as far as I am aware, a host-supplied substrate for bacterial production of a defined virulence determinant had not been identified until the work described in the following section.

Cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NANA), a host-supplied substrate for sialylation of gonococcal LPS *in vivo* which confers serum resistance

This section contains more detail than previous sections because it describes recent work and indicates how an *in vivo* situation can be probed by modern methods.

Gonococci obtained from patients and examined without subculture are resistant to complement-mediated serum killing, and this resistance is important in pathogenicity (Ward *et al.*, 1970; Parsons *et al.*, 1985). Some strains, notably those isolated from disseminated infection, retain their resistance on subculture (Parsons *et al.*, 1985). Most strains from urethral exudates do not (Ward *et al.*, 1970) and we have been trying to explain this unstable type of resistance.

The first requirement was an adequate supply of *in vivo*-grown gonococci. This could not be obtained from urethral exudates, so to begin work an animal model was essential. Gonococci were grown in subcutaneous plastic chambers in guinea-pigs. In these chambers, a laboratory strain of gonococci became resistant to killing by human serum (Penn *et al.*, 1977). Its resistance was lost after 2–3 generations in laboratory media and restored after 2–3 generations within the chambers: the changes were, therefore, phenotypic, not the result of selection of genotypes (Rittenberg *et al.*, 1977). Resistance could also be restored *in vitro* by incubation for 3 h at 37 °C with guinea-pig chamber fluid, guinea-pig serum or an ultrafiltrate of the latter in a defined medium (Parsons *et al.*, 1985). The resistance-inducing activity of the ultrafiltrate was very acid- and heat-labile (Patel *et al.*, 1984a).

Armed with this knowledge from the animal model, attention turned to the natural host. Red blood cell lysates and about 20% of tested human sera had resistance-inducing activity (Martin *et al.*, 1981, 1984; Patel *et al.*, 1984b). The relevance of the resistance-inducing factor (RIF) to gonorrhoea was investigated first. Then RIF was purified from lysates of human blood cells and identified. Its mode of action soon became apparent together with the induced gonococcal determinant of serum resistance. Finally, the determinant was demonstrated *in vivo*.

The relevance of RIF to gonorrhoea

Gonorrhoea is usually an inflammatory disease of the urogenital tract. Secretions from male and female urogenital tracts contained RIF (Martin *et al.*, 1982). So did human phagocytes, and in much greater amount than

either red blood cells or sera (Patel *et al.*, 1988). Serum samples from women suffering their first attack of gonorrhoea showed a significantly higher proportion (31%) with marked RIF activity than samples (20%) taken from uninfected controls (Martin *et al.*, 1984). Moreover, the action of RIF was not confined to the chosen test strain of *Neisseria gonorrhoeae*: it affected 30 different isolates randomly collected from clinics (Martin *et al.*, 1983).

RIF has high- M_r and low- M_r components: low- M_r RIF is CMP-NANA or a closely related compound

To follow the fractionation of RIF an assay was established. The dilutions of fractions were determined which converted 50% of serum-susceptible gonococci to resistance in 3 h at 37 °C under standard conditions; an amount arbitrarily fixed as one RIF unit (Patel *et al.*, 1984a). Ultrafiltration of serum or blood cell lysates showed two components, high- M_r RIF of M_r over 50000 (about 70% of the total activity) and low- M_r RIF of M_r 500–1000 (about 30%) (Patel *et al.*, 1984b; Nairn *et al.*, 1988). Attention concentrated on the latter (Nairn *et al.*, 1988). Like the guinea-pig material it was very acid- and heat-labile, and this property helped in its identification.

Lysates of mixed red and buffy coat cells were dialysed against Tris buffer for 24 h at 25 °C, with the diffusate being continuously recycled through a column of QAE-Sephadex A25. Active material was eluted in an NaCl gradient and desalted with Sephadex G10. It was refractionated, first on another QAE-Sephadex A25 column and then by repeated HPLC using DEAE anion exchange. Less than 500 µg of material showing one peak in HPLC and an absorbance maximum of 260 nm was obtained from 1 litre of blood. NMR by Drs J. Feeney and T. Frenkiel of the Biomedical NMR Centre, National Institute for Medical Research, London, UK, showed a mixture of components including UDP-*N*-acetylglucosamine and -galactosamine and possibly other pyrimidine nucleotides. UDP-*N*-acetylglucosamine and -galactosamine are acid-stable but a search through the Sigma catalogue revealed CMP-NANA carrying a special warning about acid lability. Nanogram quantities of commercial CMP-NANA induced gonococci to serum resistance. As expected, UDP-*N*-acetylglucosamine and -galactosamine proved inactive, as did UDP, CMP and *N*-acetylneuraminic acid (NANA). The synthetic CMP-NANA co-eluted with the preparation from blood cells in HPLC and was indistinguishable from it in patterns of acid and heat lability. The RIF activity of both materials was inhibited by CMP, which prevents sialylation reactions by CMP-NANA. The activity of the blood preparation was unaffected by neuraminidase, which also has no effect on

CMP-NANA (Gottschalk, 1972). Finally, the two materials produced identical effects on gonococcal LPS. Silver staining of LPS components in SDS-PAGE gels of proteinase K digests was less rapid and less intense than control samples after gonococci had been induced to resistance by either material (Parsons *et al.*, 1988). Thus, the blood preparation and CMP-NANA were identical by six criteria in addition to their mutual RIF activity. This is overwhelming evidence that low- M_r RIF from human blood is CMP-NANA or a closely related analogue. If the factor is CMP-NANA, relative biological activities indicate that 1 litre of blood contained about 40 µg and the most purified material about 1%. We were lucky indeed to identify low- M_r RIF.

CMP-NANA is a well-known donor of NANA to glycoproteins, carbohydrates and gangliosides under the influence of sialyltransferases (Gottschalk, 1972). It is found in many mammalian cells, including human cervical epithelial cells (Scudder & Chantler, 1981), and in some bacteria, including meningococci (Gottschalk, 1972). As far as I am aware it has not been demonstrated before in red or buffy coat cells.

The effect of CMP-NANA in inducing serum resistance was not confined to the test strain of *N. gonorrhoeae*: four randomly selected, serum-susceptible isolates were all converted to resistance (Fox *et al.*, 1990).

Mode of action of CMP-NANA: sialylation of gonococcal LPS causes inhibition of reaction with serum bactericidal antibody

The probable mode of action of CMP-NANA was apparent from known facts: CMP-NANA is a substrate for sialylation of polysaccharides and glycoproteins (Gottschalk, 1972). LPS is normally the target for bactericidal antibody (mostly IgM) of fresh human serum (Glynn & Ward, 1970; Schoolnik *et al.*, 1979; Rice *et al.*, 1980; Apicella *et al.*, 1986). Resistance or susceptibility to serum is determined by LPS structure, which varies with strain and growth conditions (Guymon *et al.*, 1982; Apicella *et al.*, 1987; Griffiss *et al.*, 1987; Stephens & Schafer, 1987). Sialylation of LPS by CMP-NANA could mask target sites from bactericidal antibody, thus producing serum resistance. In our work, we already knew that the LPS of the test strain absorbed serum bactericidal antibody (Tan *et al.*, 1986), that this LPS was changed chemically during conversion of the strain to serum resistance by low- M_r RIF from blood or CMP-NANA (see above) and that sialylation of LPS was indicated by CMP inhibiting the RIF activity of both.

Use of CMP-NANA with a ^{14}C -labelled NANA moiety proved that LPS was sialylated during resistance conversion (Parsons *et al.*, 1989). Transfer of radioactivity to bacterial LPS was detected by fluorography

following lysis, proteinase K digestion and SDS-PAGE. Omission of the proteinase K digestion showed that little, if any, radioactivity was incorporated into components other than LPS. The remote chance that the transferred radioactivity represented NANA breakdown products rather than the intact moiety was eliminated by CMP inhibiting the uptake of radiolabel, and neuraminidase treatment removed it. Hence, CMP-NANA was donor of sialyl groups to the LPS. It might also have induced new gene expression, for example a sialyltransferase. However, SDS-PAGE protein patterns of resistant and susceptible organisms were identical, and pulsing with [^{35}S]methionine during conversion to resistance by CMP-NANA and subsequent fluorography showed no *de novo* protein synthesis. Hence CMP-NANA acts solely as a substrate.

The serum resistance of gonococci which is conferred when their LPS is sialylated by CMP-NANA was largely lost when sialyl groups were removed by treating the organisms with neuraminidase for the maximum time that full viability could be retained. Hence, sialylation of gonococcal LPS is responsible for serum resistance. Furthermore, the sialylated, resistant gonococci were less able than susceptible gonococci to absorb bactericidal activity of fresh human serum (Parsons *et al.*, 1989). This indicated that LPS target sites for bactericidal antibody are masked and explains serum resistance. The masked sites are almost certainly on the bacterial surface because whole LPS liberated completely from the cell walls of resistant and susceptible organisms by proteinase K digestion absorbed bactericidal antibody equally (Tan *et al.*, 1986).

The gonococcal determinant of serum resistance: the target LPS component and the possible site of sialylation

A collaboration with American colleagues bore fruit quickly (Lesse *et al.*, 1989; Mandrell *et al.*, 1990). The LPS of *N. gonorrhoeae* is rough, i.e. its polysaccharide side-chains are short. It has different components which vary from strain to strain. A component of M_r 4500 is present in most strains and although its complete structure is unknown, reactions with monoclonal antibodies (mAbs) indicate the presence of terminal Gal β 1-4GlcNAc structures on the side-chain (Mandrell *et al.*, 1988). These structures were potential targets for sialylation by CMP-NANA because they are present on mammalian glycoproteins and are often sialylated. Two tools were available to investigate this possibility. First, strains with and without the M_r 4500 component were available. Second, two mAbs that recognized the putative Gal β 1-4GlcNAc epitope had been obtained; and also other mAbs which recognized epitopes on

lower- M_r LPS components which lacked the Gal β 1-4GlcNAc structures.

The LPS components in proteinase K digests of various strains were analysed by SDS-PAGE and immunoblotting with different mAbs. Strain 1291 contained the M_r 4500 component with the putative Gal β 1-4GlcNAc terminal structures. A pyocin-selected mutant (1291A) lacked the M_r 4500 component and had a lower- M_r LPS component without the structures. On incubation with CMP-NANA, the M_r 4500 component in strain 1291 increased in M_r by about 400; the LPS component of the mutant was unaffected.

In immunoblotting, the M_r 4500 component of strain 1291 bound the two mAbs which recognized the putative Gal β 1-4GlcNAc structure but the component of slightly higher M_r formed after CMP-NANA treatment did not. As expected, the lower- M_r LPS component of the mutant did not react with these mAbs, but its reactions with mAbs that recognized other epitopes were unaffected by CMP-NANA treatment. Eight strains which contained the M_r 4500 component incorporated radiolabel when incubated with CMP-[^{14}C]NANA, in contrast to four strains that lacked the component. In solid-phase radioimmune assays using whole organisms (three strains) as antigens, the binding of a mAb that recognized the Gal β 1-4GlcNAc structure was reduced by incubating the organisms with CMP-NANA and restored by subsequent treatment with neuraminidase. The binding of another mAb which recognized a different epitope was unaffected. Whole organisms of strain 1291 were immunogold labelled after reaction with a mAb that recognized the putative Gal β 1-4GlcNAc structure but not when they were incubated with CMP-NANA. However, the reaction with the mAb was restored by neuraminidase treatment. These studies strongly suggest the M_r 4500 LPS component is the target for sialylation by CMP-NANA and that the putative Gal β 1-4GlcNAc structure is the site of sialylation.

Recent electron microscopy of gonococci after induction to serum resistance by CMP-NANA showed dramatic surface changes (Fox *et al.*, 1990). The organisms were stained with a carbohydrate stain, ruthenium red. The majority (60–70%) of resistant gonococci showed a surface accumulation which was enhanced and on all gonococci after incubation with fresh human serum. In contrast, susceptible gonococci not treated with CMP-NANA were devoid of surface polysaccharide and remained so after incubation with heated serum, showing that serum components are not involved. The ultrastructural changes, first seen with the usual test strain, also occurred on a recent isolate. The surface material is probably sialylated LPS but this is not yet proved. Masking of the antibody-reactive sites (see above) could occur without the sialylated LPS being

visible. The surface polysaccharide may be a secondary effect of CMP-NANA treatment which occurs later in a continuous process. Such a continuous process might account for some gonococci not showing polysaccharide after incubation with CMP-NANA in the non-synchronous cultures, and the seemingly more dense polysaccharide on all the resistant organisms after further incubation with serum.

Demonstration of sialylated LPS in directly examined gonococci of urethral exudates

The scene had now been set for the clinching experiment, direct examination of gonococci from patients for the presence of sialylated LPS. Would these *in vivo* gonococci fail to react with a mAb that recognizes the putative Gal β 1-4GlcNAc structure on the M_r 4500 component *unless they were treated with neuraminidase*? This unmasking of the epitopes occurred for gonococci where LPS had been sialylated by incubating them *in vitro* with CMP-NANA (see above); and also for human infant erythrocytes, which have similar sialylated surface structures (Mandrell *et al.*, 1988).

Urethral exudates from two male patients were incubated with a mAb which recognizes the putative Gal β 1-4GlcNAc epitope on the M_r 4500 LPS component, followed by secondary antibody coupled to gold particles of one size. Then, they were treated with neuraminidase. This was followed by the same mAb but secondary antibody coupled to gold particles of a different size (Mandrell *et al.*, 1990; and unpublished observation by these authors). The different-sized gold particles enabled the reaction of the mAb before neuraminidase treatment of the gonococci to be distinguished from that after treatment. In both cases, there was little reaction of the mAb with the gonococci initially but, as for CMP-NANA-treated gonococci *in vitro*, significant binding of the mAb occurred after neuraminidase treatment. These results indicate that sialylation of the putative Gal β 1-4GlcNAc epitope of the M_r 4500 LPS component detected in experiments *in vitro* also occurs *in vivo*.

This conclusion was supported by similar experiments with gonococci that had been ingested *in vitro* by human polymorphonuclear (PMN) phagocytes. These experiments were relevant to the influence of CMP-NANA *in vivo* in two respects. Many PMN phagocytes, often containing gonococci, are found in urethral exudates. Also, human PMN phagocytes contain high resistance-inducing activity (Patel *et al.*, 1988), which is probably due to CMP-NANA or a related compound. Strain 1291, which contains the M_r 4500 component with the putative Gal β 1-4GlcNAc epitope, was incubated with human PMN phagocytes. Then, in immunogold labelling

experiments as described for the gonococci in urethral exudates, the mixture was treated with the mAb that recognizes the epitope before and after neuraminidase treatment. The usual binding of the mAb to strain 1291 was significantly decreased by 10–60 min incubation with the PMN phagocytes and restored by treatment with neuraminidase (Mandrell *et al.*, 1990).

Conclusions and future work

It appears that the serum resistance of gonococci in urethral exudates which is lost on subculture *in vitro* is due to sialylation of their LPS. The conserved M_r 4500 component seems to be involved and the site of sialylation may be putative Gal β 1-4GlcNAc structures on the side-chain. The human factor responsible for conferring resistance *in vivo* is CMP-NANA or a related compound. It acts as a substrate for the sialylation of LPS which inhibits the interaction of bactericidal antibody of human serum with its target sites.

To strengthen this view, more observations are needed on material from patients; at present sialylation of gonococcal LPS has been demonstrated only in two urethral exudates. The surface polysaccharide formed by CMP-NANA must be investigated to see if it is sialylated LPS and if there is any connection with the similar surface appearance of gonococci in urethral exudates (Novotny *et al.*, 1977) and with a 'capsule' occasionally seen in minimally subcultured gonococci (de Hormaeche *et al.*, 1978). Structural studies on the sialylated LPS will be needed to confirm the information gained by use of mAbs on the possible site of sialylation. Then, how does CMP-NANA become available to gonococci *in vivo*? Primarily, it is intracellular (Brackman *et al.*, 1983) and could, therefore, be available to gonococci in epithelial cells and phagocytes (Parsons *et al.*, 1985). The experiments with gonococci and human PMN phagocytes support this view. Only small quantities would be present in blood and body fluids because CMP-NANA does not readily cross cell membranes (Brackman *et al.*, 1983). Such quantities may, however, be biologically significant. Above all it must be remembered that the bulk of RIF in blood cell lysates is in a high- M_r form. Assuming that the high- and low- M_r RIF are connected, CMP-NANA or a related precursor may be attached to a large carrier molecule or even a sialyl transferase. Clearly, the nature of high- M_r RIF, its relation to CMP-NANA, and the availability of both to gonococci *in vivo* must be investigated.

Gonococci must contain a sialyltransferase because sialylation of LPS occurs *in vitro* in a synthetic medium and, indeed, a detergent extract of gonococci showed possible sialyltransferase activity (Mandrell *et al.*, 1990). Exogenous sialyltransferases have also been shown to

transfer NANA from CMP-NANA to gonococcal LPS components (Mandrell *et al.*, 1990). Hence, *in vivo* host sialyltransferases may sialylate gonococcal LPS. If so it would be another interesting facet of the influence of the environment *in vivo* on a pathogen.

Obviously, not all examples of serum resistance exhibited by gonococci are explained by the above results. The resistance of strains that are stable on subculture must be due to other mechanisms. Also, there are strains that contain the M_r 4500 component with the putative Gal β 1-4GlcNAc epitope and are serum resistant without CMP-NANA treatment (Mandrell *et al.*, 1990). Whether sialyl groups are involved in any of these other serum resistances remains to be seen.

Wider implications

One possible wider implication proved to have no basis. Sialylation of LPS by CMP-NANA might have been the cause of serum resistance in meningococci and other Gram-negative pathogens. However, the serum killing of various strains of *Neisseria meningitidis*, *E. coli*, *Citrobacter koseri*, *Salmonella dublin*, *Salmonella virchow*, *Salmonella heidelberg*, *P. aeruginosa* and *Klebsiella aerogenes* was not decreased by incubating them with CMP-NANA (Fox *et al.*, 1989). Also, a factor in human blood which induces serum resistance in *Haemophilus influenzae* type b is not CMP-NANA (Kuratana *et al.*, 1989).

The work has an implication for wider studies on pathogenicity. Subject to confirmation as outlined above, a host substrate, CMP-NANA, has been identified and shown to produce a defined virulence determinant, sialylated LPS, of a pathogen, *N. gonorrhoeae*, *in vivo*. This more specific look at the effect of the environment *in vivo* should be repeated for other host factors and their corresponding determinants of pathogenicity.

Final remarks

I hope that this lecture makes clear that persons interested in pathogenicity should pay attention to the microbe *in vivo*, that modern methods make this relatively easy, and that the results can be very rewarding.

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