HISTORICAL ARTICLE

A Brief History of the Pneumococcus in Biomedical Research: A Panoply of Scientific Discovery

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Because of its prominence as a cause of disease in humans, *Streptococcus pneumoniae* has been the subject of intensive investigation at both the clinical level and the basic scientific level during the past century. In a number of instances, these studies have resulted in important progress toward the comprehension of basic biological principles. The areas advanced by studies of the pneumococcus include an understanding of the concept of pathogenesis of infectious disease; the development of Gram's stain for identification of bacteria in specimens from patients; the elucidation of the role of the bacterial capsule in resistance to phagocytosis by cells of the host's immune system; the demonstration that molecules other than proteins are capable of eliciting the host's humoral immune responses and later, by extension, that isolated bacterial exopolysaccharides can be used safely and effectively as vaccines in humans; the documentation of the efficacy of penicillin; the collection of conclusive evidence that DNA encodes genetic information; and the investigation of putative proteinaceous virulence factors.

Data acquired during the course of clinical investigations can often provide the answers to basic biological questions if subjected to critical analysis by insightful researchers. Ample illustrations of this point are found in the abundant reports of investigations involving *Streptococcus pneumoniae*, an organism of unquestioned clinical importance. Since a variety of recent reviews have focused on different aspects of pneumococcal research, we will not attempt an exhaustive summary here; rather, we will direct the reader to detailed reviews where appropriate. In this review we will focus on the ways in which studies of this pathogen have been central to several of the most profoundly influential biological findings of the past 110 years.

Description of the Organism and Demonstration of Its Virulence

In 1881 two microbiologists, George M. Sternberg in the United States and Louis Pasteur in France, independently described roughly lancet-shaped pairs of coccoid bacteria in human saliva. Pasteur [1, 2] and Sternberg [3, 4] each injected human saliva into rabbits; Pasteur used saliva from a child who had died of rabies, while Sternberg used his own saliva. Both researchers subsequently recovered diplococci from the blood of these rabbits.

Previous reports identifying slightly elongated diplococci existed in the literature [5, 6], but only Sternberg and Pasteur demonstrated the pathogenic potential of these bacteria in animals. In fact, each researcher had described the same organism; it was named *Microbe septicemique du salive* by Pasteur [2] and *Micrococcus pasteuri* by Sternberg [7]. By 1886 this organism was being referred to as *Pneumococcus* by Fraenkel [8] because of its propensity to cause pulmonary disease. It was renamed *Diplococcus pneumoniae* in 1920 [9] —a designation obviously referring to pairs of cocci causing pneumonia. This epithet was first suggested by Weichselbaum in 1886 [10–13] in a series of case reports on the causative agent of what was then called croupous pneumonia; he also referred to pneumococci as "kapsel kokken." It was not until 1974, however, that the pneumococcus was given its present name, *Streptococcus pneumoniae* [14], primarily on the basis of its characteristic growth as chains of cocci in liquid media.

The causative role of this organism in human lobar pneumonia was firmly established in the early 1880s by a number of investigators [15–18]; later in that same decade, the pneumococcus was clearly demonstrated to be a cause of meningitis [19] and otitis media [20]. Robert Austrian, an influential researcher on pneumococcal vaccines and a noted historian of the pneumococcus, has written two excellent reviews on the latter subject [21, 22].

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Gram's Stain

Also during the 1880s, Christian Gram [23] was experimenting in the laboratory of Friedländer [22] with techniques for visualization of bacteria in pathological specimens. Gram examined sections of lung tissue from patients who had died of pneumonia; he exposed the specimens sequentially to aniline-gentian violet; a weak solution of iodine; ethanol; and Bismarck brown or vesuvin. Gram found that these sections contained many pairs of slightly elongated cocci that retained the dark aniline-gentian violet stain. He referred to these organisms as "the cocci of croupous pneumonia." The failure of other bacteria in Gram's specimens to retain the aniline-gentian violet demonstrated a phenomenon that would become one of the cornerstones of clinical microbiology—namely, that nearly all clinically important bacteria are either gram-positive or gram-negative. In fact (as discussed by Austrian [21]), in some of the lung sections described above, Gram saw an encapsulated bacterium that did not retain the aniline-gentian violet and that caused pneumonia (*Klebsiella pneumoniae*, or Friedländer's bacillus). This observation, had he fully appreciated it, could have forestalled an acrimonious debate between Fraenkel and Friedländer over the etiology of lobar pneumonia; in fact, each was correct [21].

The pneumococcus, therefore, was one of the first pathogenic bacteria observed during the development of Gram's stain, a bacteriologic tool that is still in everyday use more than a century after its original description.

Humoral Immunity, Bacterial Capsules, and Phagocytosis

After the early descriptions of the role of the pneumococcus in disease, Klemperer and Klemperer [24, 25] showed that serum from rabbits injected with heat-killed pneumococci or with filtrates of broth cultures contained factors that conferred immunity to reinfection with the same strain but not necessarily to infection with different clinical isolates. More important, rabbits were protected against primary pneumococcal infection by infusion of serum from a previously immunized animal [24, 25]. Issaeff [26] demonstrated shortly thereafter that this protective serum was not directly bactericidal but that it did promote uptake of pneumococci by phagocytic cells of the immune system. Earlier, the well-known immunologist Eli Metchnikoff had observed pneumococcal agglutination in antisera [27], but he apparently did not make the connection between this agglutinating factor and the promotion of phagocytosis. This point is ironic, in that Metchnikoff was the first to describe the phenomenon of phagocytosis. In any event, *S. pneumoniae* was the organism used to document the protection of animals by active immunization and the presence of the protective factor in serum.

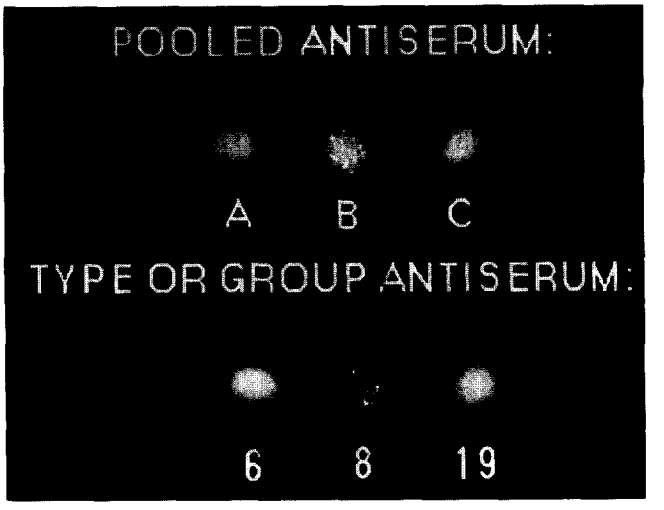

Figure 1. *Upper panel:* Reactivity of *S. pneumoniae* serotype 8 (ATCC 6308) with antiserum pools A, B, and C. Specific agglutination is evident for pool B, which contains antibody specific for serotypes 3, 4, and 8, and group 19. *Lower panel:* Specific reactivity of the same strain with antiserum to serotype 8 polysaccharide. Pneumococci were stained with ethidium bromide, washed twice with PBS, resuspended at a concentration of $\sim 5 \times 10^7$ cfu/mL, and mixed 1:1 with the indicated antisera.

At the turn of the century, Neufeld demonstrated both macroscopic agglutination and microscopically visible, specific swelling (*quellung* in his native German) of the external capsule upon the addition of specific antiserum to a suspension of pneumococci [28]. For most pneumococcal serotypes, homologous rabbit polyclonal antiserum mixed in equal parts with a cloudy suspension of bacteria ($\sim 10^7$ cfu) results in macroscopically visible bacterial clumping (figure 1), thereby providing a simple method of serotyping. Bile solubility testing, the quellung and agglutination reactions, and additional techniques are lucidly discussed in a classic review of the laboratory identification of pneumococci by Lund [29].

The apparent discrepancy between humoral and cellular immunity was resolved in 1904, when Neufeld and Rimpau [30] showed that ingestion of pneumococci by white blood cells was greatly facilitated by preexposure of the bacteria—but not the white cells—to serum from a previously immunized animal. The phenomenon they demonstrated was what we now call opsonization (from the Greek word for "preparing food"), in which the coating of bacteria with complement components and immunoglobulins leads to Fc receptor–mediated uptake by phagocytic cells.

Definitive proof of the critical importance of the capsule to virulence was established in a pair of papers printed back to back in the *Journal of Experimental Medicine* in 1931. In the first paper Rene Dubos and Oswald Avery showed that an enzyme obtained from a soil bacillus removed the serotype 3 capsular polysaccharide [31]. In the second paper [32] these

investigators demonstrated the protection of mice by the enzyme against otherwise-fatal challenge with *S. pneumoniae* serotype 3. This enzyme was later shown by Francis et al. [33] to provide the same protection to the Java monkey.

It is clear from the foregoing discussion that, even without knowledge of the specific structure or mode of action of antibodies, early investigators were well aware of the presence and importance of this serum component. Less well studied in the early 1930s was the possible role of nonimmunoglobulin serum molecules in opsonophagocytosis of pneumococci. While Ward and Enders [34] first demonstrated the necessity for such a factor in 1933, little additional work was completed until 1969, when Johnston et al. [35] outlined the effect of complement in increasing the rate of pneumococcal phagocytosis. Winkelstein and (later) Hosea, Brown, and other researchers specifically delineated the locations and mechanisms of activation of the classical and alternative pathways of complement by encapsulated pneumococci, leading to phagocytosis (see [36] for references). These studies helped to clarify the relative contributions of immunoglobulin and complement opsonins to the opsonophagocytosis of encapsulated pathogenic bacteria. The subject has been reviewed by Winkelstein [36] and—quite recently—by Janoff et al. [37] as part of a broader discussion of pneumococcal disease during infection due to human immunodeficiency virus.

The Concept of Serotyping

The discovery that the injection of pneumococci into rabbits had an immunizing effect facilitated the development of an elementary typing system for this bacterial species. Neufeld and Haendel [38] classified isolates from patients with confirmed pneumococcal pneumonia into two groups on the basis of whether or not they killed mice previously immunized with pneumococcal isolates referred to as type I or type II. The authors correlated these results with those obtained in agglutination reactions. Three years later Dochez and Gillespie [39] extended these groupings to include three distinct pneumococcal serotypes as well as a fourth group that was heterogeneous. All isolates of the first three serotypes reacted with antiserum to any other organism of the same serotype. In contrast, each member of the fourth cluster failed to react with antisera to the first three serotypes but instead tended to react only with antiserum produced by immunization of a rabbit with that specific isolate. Lister [40, 41], working in South Africa, confirmed the validity of this typing system and showed that virulent strains unrelated to the American strains studied by Dochez and Gillespie existed in South Africa.

It is worth noting that the third pneumococcal serotype to be established was phenotypically distinct from types 1 and 2 and from the group 4 isolates; when grown on solid agar, it produced colonies that were noticeably larger, more mucoid, and more iridescent than those produced by serotype 1 or 2 or by group 4. In fact, for some time, what we now refer to as *S. pneumoniae* serotype 3 was considered to be a separate species known as *Pneumococcus mucosus* [42]. It is now known, however, that serotype 37 also exhibits a highly mucoid phenotype and thus is macroscopically indistinguishable from serotype 3 on blood agar plates [43]; moreover, on rare occasions, we have observed this phenotype among clinical isolates of serotypes 6A and 19F (authors' unpublished observations). The detection of recurring reactive types (serotypes) among group 4 pneumococci eventually led to the identification of 85 distinct serotypes [44], largely through the efforts of Cooper, Eddy, Morch, and Lund before 1960. Lund [29] beautifully reviewed the history of these studies. An excellent review of the immunogenicity and immunochemistry of pneumococcal capsular polysaccharides has recently been published by van Dam and associates [44].

Polysaccharides as Capsular Material

While working at the Rockefeller Institute in New York City in 1917, Dochez and Avery [45] described a soluble specific substance they had found in serum and urine from patients with lobar pneumonia and in blood from animals experimentally infected with pneumococci; this substance formed a precipitate with specific antiserum to the homologous pneumococcus. By identifying this substance—which comprised the pneumococcal cell envelope—as a complex carbohydrate or polysaccharide, Heidelberger and Avery [46] unambiguously established that the capsular polysaccharide was the factor responsible for serological reactivity. Of the pneumococcal cell, Heidelberger later concluded [47]: "[T]here is disposed at its periphery a highly reactive substance upon which type specificity depends." Heidelberger and colleagues further showed that this capsule was antigenic; that is, the complex carbohydrate composing this covering induced immunity in mice that protected these animals from lethal infection upon subsequent pneumococcal challenge. Before this seminal observation was reported, it had been widely believed that only proteins were capable of eliciting an immune response [48].

Vaccine Studies

Even before the demonstration of the immunogenicity of the bacterial capsular polysaccharide, studies begun in 1911 by Sir Almroth E. Wright and colleagues [49]—with South African gold miners as test subjects—suggested that inoculation of whole killed pneumococci might elicit protection against pneumococcal infection in human beings [50]. In this work Wright followed the principles of study he had already used with reasonable success in vaccinating subjects against typhoid fever [51]. Unfortunately, the results he obtained with pneumococcal vaccine did not convince the sci-

entific community of its efficacy. The problem lay in the failure to include both pneumococcal serotypes known at that time and in the use of an inadequate vaccine dosage [22] because of the discomfort associated with the injection of relatively large inocula of whole killed pneumococci.

In 1926 Felton and Baily [52] described the separation of capsular polysaccharides and showed that the resulting material, called "soluble specific substance," was the subcellular fraction responsible for conveying immunity. This work opened the door for Francis and Tillett [53] and Finland and co-workers [54–57] to conduct a number of studies (during the 1930s and 1940s) of the effectiveness of vaccines aimed at the prevention of pneumococcal disease. In 1937 Felton's capsular material was used successfully in a program of mass vaccination to abort an outbreak of pneumonia at a state hospital [58]; this was the first instance in which active vaccination with a relevant subcellular bacterial fraction had been used for such a purpose. Besides Finland, other pioneers in the field at this time included Felton himself [59], MacLeod and colleagues [60], and Heidelberger and associates [61]; each investigator or group of investigators showed that healthy adult volunteers were protected against pneumococcal infection by vaccines that stimulated the immune system to produce antibodies to the pneumococcus. Kaufman [62] demonstrated that pneumococcal vaccines containing two and later three type-specific polysaccharides (i.e., bivalent and trivalent vaccines) were efficacious in an elderly cohort. These studies led to the licensing of hexavalent polysaccharide vaccines for human use after World War II. However, these vaccines were not used by physicians at that time because many believed that newly available drugs constituted a more effective means of dealing with pneumococcal disease; as a result, the vaccines were withdrawn from the market [22].

Interest in pneumococcal polysaccharide vaccines was revived in the mid-1960s, largely because of the efforts of Robert Austrian. Work on a multivalent vaccine containing the polysaccharide components of each of the 14 most common pneumococcal serotypes (which caused some 80% of cases of pneumococcal disease) began in 1967 and culminated in the introduction of a 14-valent vaccine in 1977. This advance followed studies by Austrian et al. [63, 64] in which such a vaccine was efficacious in certain populations with high attack rates of pneumococcal pneumonia. A 23-valent vaccine containing an even larger percentage of the pneumococcal serotypes commonly causing disease was introduced in 1983 [65] and is the subject of recent reviews [66, 67] and comment [68]. A number of studies have evaluated the efficacy of this vaccine [69–71], and all have yielded values in the range of 55%–65%. The most recent of these reports also showed that the age and immune status of the patient as well as the interval since vaccination all figure significantly into the equation [72]. The degree of efficacy has not been uniform in all populations, however, with particularly low success rates among very young children [73], debilitated elderly persons [74], or individuals whose immune systems are compromised [72, 75]; production of a vaccine that is efficacious in these high-risk groups remains a cherished but elusive goal. Pneumococcal polysaccharides of several serotypes have been conjugated to carrier proteins [76–79], a technique previously used with great success for *Haemophilus influenzae* type b polyribosyl ribitol phosphate. A number of clinical trials of pneumococcal conjugate vaccines are in progress; to date, the results have been generally favorable [72], though not unequivocally so (D. M. Musher, M. C. Rodriguez-Barradas, J. E. Groover, and D. A. Watson, unpublished observations). While much remains to be accomplished in this area, Broome and Breiman [68] point out that the current 23-valent vaccine can greatly reduce the number of cases of bacteremic pneumococcal infections and should therefore be more widely administered to the persons for whom its use is indicated.

Chemotherapy

In 1911 Morganroth and Levy [80] showed that a quinine derivative, ethylhydrocupreine (also known as optochin), inhibited the growth of pneumococci but not of clinically related organisms. The use of optochin by Morganroth and Kaufmann [81] to treat experimentally infected mice is one of the first examples of the use of a specific antimicrobial agent as therapy for a serious bacterial infection—and, in fact, of *any* highly specific compound as therapy for *any* infection. Quinine had previously been evaluated for the treatment of pneumococcal pneumonia in humans [50]; the minimal success of this effort contrasted with the great importance of quinine in the treatment of malaria. Morganroth and Kaufmann showed that pneumococci rapidly became resistant to clinically achievable doses of optochin, possibly through the acquisition of a single point mutation, as our recent data suggest [82]. In addition, optochin had only a narrow window of effectiveness between therapeutic and toxic dosages [83]; its use was rapidly abandoned due to its optic toxicity [84].

Serotherapy

During the 1930s two important new approaches to therapy were developed, at least in part through their application to pneumococcal infection. The first approach—the infusion of pneumococcal antiserum produced in animals for the treatment of active pneumococcal infection in humans [85] —had been shown much earlier to be effective in animals [24, 25]. In the last decade of the nineteenth century, numerous investigators had obtained mixed results with immune serum from a variety of animal sources [50]. Interest in this approach was probably fueled by the successful reduction in mortality from diphtheria by the same basic technique. However, the underlying principle was quite different in the latter

case: serotherapy for diphtheria involved an antiserum to the toxin, whereas serotherapy for pneumococcal infection was aimed at the transfer of antibody that would opsonize the infecting organism and therefore eradicate it from the host. It was not until the 1920s—when serotypes began to be recognized, when antisera were standardized according to serotype, and when sera from repeatedly sensitized horses were used—that consistently good results were first reported [86]. Sera from patients who had recovered from pneumonia were theoretically preferable to those from animals because of the reduced risk of serum sickness; unfortunately, the potency of these preparations of human serum was inferior, and their use was abandoned [87].

Use of Antimocrobial Agents

The second new approach to therapy was the administration of defined chemotherapeutic agents—first sulfanilamide and later penicillin.

Sulfanilamide. Among the earliest uses of the antimicrobial compound sulfanilamide was that for the treatment of pneumococcal pneumonia, although the frequency with which this option was selected was limited by the popularity of serotherapy [51]. Since the pneumococcus did not exhibit the same extreme susceptibility to sulfanilamide as did *Streptococcus pyogenes*, Whitby [88] undertook a systematic search for a related chemical compound with good in vitro activity but relatively low toxicity. According to this author, "these experiments represent the one striking success in the chemotherapy of pneumococcal infections in an assessment of no less than 64 related sulfanilamide compounds." One of these 64 derivatives possessed the proper combination of low toxicity and good in vitro activity; this compound was referred to as 2-(*p*-aminobenzenesulfonamido) pyridine, or simply sulfapyridine. (Whitby's approach has been the basis, in more modern times, for selection of a particular formulation of a given antimicrobial compound for further clinical testing.) This work [88] was followed only 5 weeks later in *The Lancet* by the study of Evans and Gaisford [89], who reported that treatment with sulfapyridine reduced the overall case-fatality rate from 27% to 8% among patients with pneumonia (including 100 with lobar pneumonia) at the Dudley Road Hospital in Birmingham, England.

Thus, for a brief period, sulfapyridine appeared to be the treatment of choice for pneumococcal infections. By 1943, however, in an early example of an increasingly important problem, sulfonamide-resistant strains of *S. pneumoniae* were reported by Tillett et al. [90].

Penicillin. In 1929 Fleming [91] discovered the antibacterial properties of the fungus-derived substance that came to be called penicillin. The third subject to receive this drug (by topical application)—and the first to show any clinical benefit—was suffering from pneumococcal conjunctivitis [51]. Compared with sulfanilamide, penicillin possessed a number of superior attributes, including greater potency per unit, minimal influence of inoculum size on effectiveness, and lack of interference by the breakdown products of protein hydrolysis [92]. However, the efficacy of readily synthesized sulfanilamide in treating pneumococcal infections, coupled with the difficulty of obtaining sufficient quantities of penicillin, meant that the full potential of the latter drug in combating the pneumococcus was not immediately realized.

In 1939 Dubos [93] discovered the first naturally occurring antimicrobial compound with demonstrable activity in vitro against a bacterial pathogen. This compound was named gramicidin, and the activity demonstrated was against *S. pneumoniae.* Unfortunately, like optochin, gramicidin proved to be toxic in mice [94] and dogs [95], and this toxicity effectively ruled out its use in humans. On the positive side, however, the identification of this compound by Dubos did prompt Chain and colleagues [96] to reevaluate the antibacterial properties of penicillin in 1940. This reanalysis was made possible by methods that these investigators developed at Oxford for the isolation of penicillin in large quantities and for the rapid assay of its inhibitory power, as described in detail by Abraham et al. [92] in a landmark paper appearing in *The Lancet* in 1941. In the same elegant paper, this group detailed their dramatic results in the treatment of life-threatening infections caused by gram-positive cocci, including *S. pneumoniae.* As a result, the approach to the treatment of pneumococcal infections was changed forever. In 1943 Keefer et al. [97] reported a series of 500 cases in which penicillin was used with great success in the treatment of a variety of staphylococcal and streptococcal (including pneumococcal) infections, mostly those resistant to sulfonamides. According to a personal communication from Louis Weinstein:

> Having obtained small amounts of penicillin through his contacts with researchers at Oxford, Dr. Chester Keefer first tried to treat patients at the Boston Memorial Hospital with 5,000 units of penicillin every four hours for viridans streptococcus endocarditis. When that treatment failed, Keefer (the supervisor) and [I] (the acting intern) turned to the treatment of pneumococcal pneumonia with dramatic results. This was the first disease for which penicillin was used successfully in the United States.

The next year, Tillett et al. [98] reported on the use of penicillin in 46 cases of pneumococcal pneumonia and 8 cases of pneumococcal empyema, again with excellent results. This study was useful in further defining proper treatment for pneumococcal infections, since the Keefer study—conducted during a period of great dedication to the U.S. effort in World War II—was focused narrowly "toward those infections that are most likely to occur in our armed forces." These investigations yielded convincing evidence of the value of penicillin in the treatment of a variety of bacterial infections. As Mufson has pointed out [99], studies of

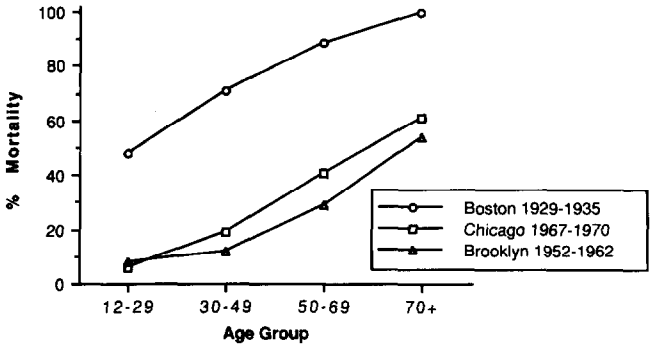

Figure 2. Graphic representation of data taken from Mufson [99], showing a uniform decline of 40%–50% in mortality associated with pneumococcal bacteremia for every age group after the introduction of penicillin for the treatment of pneumococcal infection. Data for mortality due to pneumococcal bacteremia in the era preceding the introduction of penicillin were obtained at the Boston City Hospital, while those for mortality after the introduction of this drug were obtained at Kings County Hospital in Brooklyn, New York (1952–1962), and at Cook County Hospital in Chicago (1967–1970).

mortality from serious pneumococcal infection as a function of age showed a dramatic reduction after the introduction of penicillin (figure 2).

Studies of the pneumococcus were among the first to document the clinical relevance of penicillin-binding proteins (PBPs) in the development of resistance to penicillin. Close examination of the data from the paper published in *The Lancet* in 1941 by Abraham et al. reveals that even the first studies of the in vitro susceptibility of *S. pneumoniae* to penicillin detected a biphasic pattern. Specifically, one group of pneumococci was at least 30 times as susceptible to the drug as was the other, yet both groups included isolates of the same serotypes. In fact, serotype 19F, recently associated with both moderate and high-level resistance to penicillin, was originally identified by Abraham et al. [92] as being the serotype of one of the less sensitive isolates. Although to our knowledge the PBP profiles of these strains have never been examined, it is likely that differences in PBPs were responsible for the discrepancy. By 1943 it had been shown that pneumococcal resistance to penicillin could be induced in vitro [100] or in vivo (in the mouse) [101]. In light of these findings, the reports by Hansman and Bullen [102] of a highly penicillin-resistant pneumococcus and later by Appelbaum et al. [103, 104] and by Jacobs et al. [105] of a large outbreak of penicillin-resistant pneumococcal infections are surprising, not so much because penicillin-resistant clinical isolates of *S. pneumoniae* were identified but because such isolates took so many years to appear.

The mechanism by which resistance to penicillin arises in pneumococci has been shown to be decreased binding of the drug to PBPs, which are also known as transmembrane carboxypeptidases—enzymes involved in cell wall synthesis. (See Waxman and Strominger [106] for a review.) The concept of such surface proteins was developed by Spratt [107] in studies with *Escherichia coli;* pioneering investigations of decreased penicillin binding to pneumococci were published in 1954 by Eagle [108, 109], albeit without knowledge of specific surface-associated PBPs. The large-scale outbreak of penicillin-resistant pneumococci in South Africa discussed above [105] led to the identification of one of the first clinical correlates of the PBP concept—namely, that alteration of these proteins contributed to the development of resistance to penicillin. In 1980 Hakenbeck et al. [110] reported alterations in the PBPs of clinical isolates of pneumococci associated with increased resistance to penicillin. Zighelboim and Tomasz [111] extended this finding to penicillin-resistant isolates from South Africa and further described the mechanism of resistance. Additional studies of PBPs have been reported by Hakenbeck et al. [112, 113], Chalkley and Koornhof [114], Dowson and colleagues [115], and Jabes et al. [116]. Moreover, in two of only a few well-documented instances, pneumococci have been shown to be both the donors of altered PBP DNA sequences to other streptococcal species [117] and the recipients of such sequences from another streptococcal species [118]. Horizontal transfer of PBP genes has also been demonstrated among natural populations of pneumococci [119, 120], and penicillin-resistant clones have even been shown to have taken transcontinental journeys [121]. The increasing frequency of penicillin-resistant pneumococci [122–124] is especially worrisome in light of the trend toward higher levels of resistance to vancomycin among enterococci, since horizontal genetic transfer of the latter resistance to pneumococci seems likely.

Discovery of the Transforming Principle

In 1916 Stryker [125] described changes that occurred in pneumococci upon growth in broth containing homologous immune serum. She noted that, when virulent strains were cultured in this fashion, they became less virulent, produced less capsular material, were more readily ingested by phagocytes, and displayed altered antigenic properties. Griffith built on these data [126], borrowing the terminology of Arkwright [127] to describe the appearance of colonies of dysentery bacilli on plates containing homologous immune antiserum. Smooth ("S") colonies, as defined by Griffith [128], possess a lustrous, mucoid, macroscopically apparent colonial phenotype attributable to the presence of a polysaccharide capsule; agglutinate in the presence of homologous antisera; cause fatal infections in laboratory animals; and, when injected into rabbits, stimulate the production of protective antibodies. Rough ("R") forms do not possess the extracellular polysaccharide capsule; are avirulent; and, when injected into rabbits, lead to the production of antisera specifically reactive only with other rough pneumococci.

Griffith showed that some induced rough forms could re-

vert to the smooth form in vivo, while others could not. Even rough forms that never spontaneously reverted to capsule production, which he regarded as completely "dissociated" (i.e., unable to produce a capsule), could be transformed back to their original capsular types by a novel technique that Griffith himself pioneered. This procedure involved the concomitant injection into mice of heat-killed smooth pneumococci of the same or a different capsular type together with the nonrevertible rough strain. Under these conditions Griffith found that the rough form not only could be made to revert to its original capsular type but also could acquire the capsular type of the heat-killed organism. He did not understand the significance of this finding at the time, but his results were quickly verified by Neufeld and Levinthal [129]. Dawson and colleagues [130–133] and Alloway [134, 135] appreciably extended these observations. Alloway demonstrated the phenomenon in vitro, using extracts of *S. pneumoniae*; in his first study he dissolved the bacteria by repeated freezing and thawing, while in the second he added sodium deoxycholate to bacterial suspensions for lysis. Tragically, the scientific careers of both Griffith and Neufeld—the former British and the latter German—were cut short by incidents directly related to World War II [136].

It was not until 1944 that a landmark (and at that time controversial [136]) paper was published by Avery, MacLeod, and McCarty [137]. Their studies showed conclusively that DNA—and not some other molecule—constituted the genetic material responsible for phenotypic changes during transformation. In a now-famous letter to his brother Roy [138], Oswald Avery was cautiously optimistic:

> . . . [A]t last *perhaps* we have it. . . . [T]his [fibrous] substance is highly reactive and on elementary analysis conforms *very* closely to the theoretical values of pure desoxyribose nucleic acid (thymus) type (who could have guessed it).... If we are right, and of course that is not yet proven, then it means that nucleic acids are not merely structurally important but functionally active substances in determining the biochemical activities and specific characteristics of cells and that by means of a known chemical substance it is possible to produce predictable and hereditary changes in cells. This is something that has long been the dream of geneticists.

This observation later was strongly supported in two ways. First, after publication of the 1944 paper, McCarty, while still working in Avery's laboratory, showed that "treatment of the transforming principle with concentrations of DNase (a partially purified pancreatic enzyme which is capable of depolymerizing DNA) so small that only a slight fall in viscosity (of the DNA solution) occurs causes a marked loss of biological activity" [139]. This finding constituted further proof of the nucleic acid nature of the transforming principle. In a brilliant foreshadowing of the future work on DNA by Watson and Crick, McCarty stated: "It remains one of the challenging problems for future research to determine what sort of configurational or structural differences can be demonstrated between desoxyribonucleates of separate specificities" [139]. Later, Hotchkiss [140] showed that, in addition to the genes encoding capsule production, those sequences specifying resistance to the powerful antibiotic penicillin could be transferred to a previously penicillin-sensitive pneumococcus by DNA isolated from a penicillin-resistant pneumococcus. Although Hotchkiss joined Avery's group in 1935, he did not become involved in the work on *S. pneumoniae* transformation work until 1946, when (as recalled in an unpublished speech by Dr. Maclyn McCarty nominating Dr. Hotchkiss for an honorary doctorate in humane letters at the Rockefeller University in 1988) he

> quickly made a number of advances that clarified the transforming reaction and addressed the criticism that the apparent activity of the transforming DNA must be due to contaminating protein (as suggested by Alfred Mirsky).... [Hotchkiss] answered the challenge of contaminating protein by further purification of the pneumococcal DNA without loss of activity until only minute traces of protein remained. In other experiments initiated at this time, he broadened the genetic implications of transformation by showing that traits other than capsule formation (e.g., antibiotic resistance) could be introduced by the transfer of DNA. . . . As a result, all but the most hardened skeptics were convinced that DNA is the bearer of genetic information.

In this instance, possibly as never before, the pneumococcus was at center stage in a critically important scientific discovery; one that in fact initiated the era of molecular biology and is arguably one of the single greatest achievements in biological science in the twentieth century. It is known that Hershey and Chase, who performed the classic experiment showing that infecting bacteriophages inject only DNA into their bacterial targets (an event that results in the production of progeny phages), were inspired by their knowledge of Avery's paper [48]. Moreover, Watson stated in two different passages of *The Double Helix*, the best-selling account of the discovery of the structure of DNA, that both he (through his mentor, Salvatore Luria) and Francis Crick became convinced that DNA was the genetic material by reading the paper by Avery et al. [141].

While Avery and associates showed that the transforming principle actually encoding the encapsulation phenotype of *S. pneumoniae* consisted exclusively of DNA, the genes responsible for capsule production in the pneumococcus have never been cloned. In 1959 Austrian and colleagues [142, 143] showed that DNAs that were obtained from unencapsulated derivatives of two pneumococcal serotypes and that contained separate mutations in a common biosynthetic pathway could complement each other and produce "binary capsulation." The most important conclusion to be drawn from this work was that "the capsular genome appears to have a specific location in the total genome of the cell, this location being occupied by the capsular genome of whatever

capsular type is expressed by the cell... [and] the new capsular genome is transferred to the transformed cell as a single particle of DNA" [143]. Ravin [144] demonstrated the same concept in the same year—namely, that DNA involved in encapsulation consists of a discrete contiguous unit, or cassette. Coffey et al. [120] have recently presented indirect evidence for horizontal transfer of encapsulation genes to a penicillin-resistant pneumococcus in nature. The genetics of the encapsulation process has been investigated in a few other bacterial species, with *E. coli* K12 [145–147] and *H. influenzae* type b [148–151] most extensively studied. A sequence putatively involved in encapsulation of *S. pneumoniae* serotype 3 has recently been targeted [152] and cloned in our laboratory [153] and is currently the subject of intensive DNA sequencing efforts. Preliminary data suggest the presence of at least two loci: one unique to each serotype and one common to all serotypes.

Proteins as Virulence Factors

In recent years considerable effort has been directed to the question of whether accessory proteinaceous virulence factors exist in the pneumococcus, as they do in many bacteria, including some streptococci. Boulnois [154] has extensively reviewed a number of putative proteinaceous virulence factors of the pneumococcus, two of which deserve mention here since they have been the subject of much recent work. The first is the sulfhydryl-activated but nonsecreted hemolysin referred to as pneumolysin [154]. During the 1980s a number of studies (see [154] for references) convincingly showed that pneumolysin alone can produce all the manifestations of pneumococcal pneumonia. Since pneumolysin is liberated upon autolysis of pneumococci, it is not difficult to visualize a role for this toxin in disease. Pneumolysin may, in fact, eventually be shown to be the elusive toxin long considered a major contributor to the morbidity and mortality associated with pneumococcal pneumonia. It remains to be seen, however, whether the amount of toxin produced per bacterial cell varies among strains. Such variation could begin to explain observed differences in virulence among strains of the same serotype ([155] and authors' unpublished observations). The surface-associated protein pspA [156, 157] may also serve a still-unidentified function in virulence, given that isogenic pspA strains of some (but not all) serotypes examined to date exhibit greatly reduced virulence [158].

Future Trends in Pneumococcal Research

Predicting the future is not a science, even when science is the subject under discussion. However, the study of what Avery called the sugar-coated microbe has yielded a number of unexpected and profoundly important basic biological discoveries (as outlined herein), and we are convinced that the jigsaw puzzle of pneumococcal pathogenesis will continue to attract investigators whose efforts will yield results with broad implications.

Research on improved polysaccharide-protein conjugate vaccines will continue to be an area of great interest over the next several years, since a vaccine that is efficacious in very young children and other high-risk groups remains a high priority. Elucidation of the molecular basis for capsule production among pneumococci is a field in which we and other researchers are presently quite involved. The recent identification of short (154-base-pair) repeated-sequence elements strategically located with respect to virulence genes and to metabolically important genes in the *S. pneumoniae* genome [159] immediately suggested to their discoverers the possibility of coordinated regulation of important genes by these elements. Elucidation of the molecular machinery of such a control mechanism could add much to our understanding of pneumococcal pathogenesis.

The trend toward an increased incidence of penicillin-resistant pneumococci shows no signs of reversing and is particularly alarming in some locations. As has been discussed, resistant clones in the nasopharyngeal cavities of colonized travelers are probably being disseminated from continent to continent. If resistance to vancomycin can be passed from enterococci to pneumococci via horizontal gene transfer, we may soon see multidrug-resistant pneumococcal infections that are virtually untreatable. New antibiotics and different therapeutic strategies obviously need to be developed. Concurrent administration of new types of nonsteroidal anti-inflammatory drugs and antibiotics may be promising for the treatment of pneumococcal meningitis.

Given this wealth of possibilities, the future of pneumococcal research promises to be at least as exciting as its past.

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