Review Article

Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*

GRAHAM J. BOULNOIS*

Department of Microbiology, Medical Sciences Building, University of Leicester, Leicester LE1 9HN, UK

Introduction

Streptococcus pneumoniae is an important agent of disease in man at the extremes of age and in individuals with underlying debilitating conditions. It is responsible for the majority of cases of community-acquired pneumonia, an important cause of septicaemia, one of the three most common pathogens in bacterial meningitis and the most prevalent agent in otitis media (Roberts, 1985). The precise incidence of each of these infections is not known because of problems in diagnosis (particularly with respect to pneumonia and otitis media) and inadequate reporting of cases. However, Austrian (1982*b*), having reviewed a number of studies, concluded that in the USA there are 2–5 cases of pneumonia per 1000 population per annum, equating to between 440 000 and 1.1×10^6 cases each year. Despite antibiotic therapy the case fatality rate is about 5–7%, making pneumonia one of the major causes of morbidity and mortality in the developed world. In the same study, Austrian estimated that the attack rate for septicaemia was between 25 and 50 per 100 000 population per annum, with a case fatality rate of between 17 and 24%. Most, but not all, of these cases were associated with lower respiratory tract infections. In an attempt to focus attention on the desirability of developing an effective vaccine to protect against pneumococcal infection, this situation was likened, in terms of incidence and mortality, to that of poliomyelitis prior to the introduction of the vaccine. The incidence of pneumococcal meningitis is estimated to be about 1.5 per 100 000 population per annum (Austrian, 1982*b*). It seems likely that similar attack and fatality rates are

found in Europe. As may be expected, pneumococcal infection in the Third world poses enormous problems, with attack rates of over 10 per 1000 population per annum (Greenwood, 1987).

Both bacteraemia and meningitis can be associated with pneumonia. In untreated infections, the co-incidence of pneumonia and bacteraemia (about 30% of cases), and pneumonia and meningitis (about 2% of cases at post mortem), varies with serotype and the population studied (Heffron, 1939). In one study, more than 50% of fatal cases of untreated pneumonia were associated with bacteraemia and in some groups 10.5% of fatal pneumonias were accompanied by meningitis (Heffron, 1939).

Pneumococcal carriage and disease

Although the pneumococcus is an important cause of disease, its normal ecological niche is the nasopharynx of man and occasionally animals. It is likely that the entire human population is colonized by the pneumococcus at one time or another, and that at a given time up to 60% of individuals may be carriers (Austrian, 1986). Simultaneous carriage of more than one serotype is well documented, and this can be reflected in mixed infections (Austrian *et al.*, 1977). In general, the rate of disease within a population depends on the frequency with which invasive serotypes are carried in the nasopharynx (Riley & Douglas, 1982). However, in certain populations some serotypes are rarely carried but are frequently isolated from cases. These serotypes may be considered as particularly virulent, with the carrier state being at best a transient state. Conversely, some serotypes are frequently carried yet rarely cause disease and some serotypes show a propensity for persistence in healthy carriers. Interestingly, infection is more likely to result following the recent acquisition of a given

*Tel. (0533) 522944; fax (0533) 523013.

Abbreviations: CRP, C-reactive protein; PMNL, polymorphonuclear leucocyte; PspA, pneumococcal surface protein A; SLO, streptolysin O; TNF, tumour necrosis factor.

pneumococcal strain rather than an extended period of carriage. Nasopharyngeal carriage of pneumococci by man is very often accompanied by the development of protection to infection by the same serotype. Carriage rates are inversely related to age and the levels of anticapsular antibody and thus it is clear that the immune status of the host is an important determinant of the prevalence and longevity of carriage (Gwaltney *et al.*, 1975). *A priori* it is reasonable to suppose that pneumococcal factors also influence carriage and there is circumstantial evidence to support this suggestion, although very little is known of the pneumococcal carrier state in terms of the host-microbe interaction.

For many bacteria which colonize the mucosal surfaces the production of specific factors which are thought to mediate attachment of bacteria to epithelial cells is commonplace. It has been suggested (Andersson *et al.*, 1983) that pneumococci adhere to cells via an interaction with GlcNAc β 1-3Gal. However, another study (Krivan *et al.*, 1988) indicated that pneumococci bind to GalNAc β 1-4Gal and in this regard resembled other bacteria which exhibit a tropism for the upper respiratory tract. GalNAc β 1-4Gal is found in a number of glycosphingolipids and one of these, asialo-GM1, is found in substantial amounts in human lung tissue. The nature of the pneumococcal adhesin is not clear, although a protein-like molecule has been described which acts as a sandwich to bridge a pneumococcal cell wall component and the target receptor on epithelial cells (Andersson *et al.*, 1988). The same authors point out the similarities between the sandwich adhesin and the competence factor of the pneumococcus, which is involved in natural transformation. Pneumococcal isolates from cases of otitis media were found to be more proficient at attachment to human pharyngeal epithelial cells than those from septicaemia and meningitis (Andersson *et al.*, 1981). This may indicate that expression of adhesive capacity is modulated by the *in vivo* environment.

Other pneumococcal factors which may be involved in colonization include neuraminidase. This enzyme cleaves sialic acid from glycolipids and gangliosides and has been suggested to expose receptors for pneumococcal attachment (Krivan *et al.*, 1988). This may explain why attachment of *S. pneumoniae* to respiratory epithelial cells is enhanced following infection with the neuraminidase-producing influenza virus (Plotkowski *et al.*, 1986) and perhaps, in part, account for the prevalence of pneumococcal disease following influenza infection. Pneumococci also produce an IgA1-specific protease (see below) which may act to compromise host defence at the mucosal surface (Kilian & Reinholdt, 1986). Similarly the properties of pneumolysin described below indicate that it might also serve to abrogate host defence in the

nasopharynx and facilitate colonization. Although many of these factors have been considered as virulence factors (see below), it is not unreasonable to suggest that their primary role is in promoting carriage and that their virulence potential is only manifest when bacteria gain access to normally restricted sites.

Pathogenesis of pneumococcal infection

The processes involved in the translocation of pneumococci from the nasopharynx to other sites, including the lung, are poorly understood and probably multifactorial (Busse, 1991, Johnston, 1991).

Given the prevalence of the carrier state and the relative rarity of pneumococcal infection it is apparent that at any one time most individuals are protected from pneumococcal disease. It has been generally accepted for many years that pneumonia results from the inhalation of pneumococci from the upper respiratory tract, although in some animal models a blood-borne route of dissemination from the upper respiratory tract has been described (Rake, 1936). A multitude of specific and non-specific defences protect the lower respiratory tract. Important non-specific mechanisms include filtration, cough reflexes, secretions and mucociliary transport (Busse, 1991). Many events can compromise these defences, including sleep and agents that affect the level of consciousness (anaesthesia, drug and alcohol abuse). Viral infection of the respiratory tract also disrupts non-specific defences of the lung, and the association between influenza and pneumococcal pneumonia is well documented. Humoral and cellular components of host defence are also important in the respiratory tract (Busse, 1991). Antibody and complement components which are particularly prevalent in the alveolar lining fluid act as opsonins to facilitate phagocytosis. The resident alveolar macrophage is likely to be a key cell in this regard, at least initially, although complement cleavage products as well as macrophage and bacterial products also act to recruit polymorphonuclear leucocytes (PMNLs). Once ingested, pneumococci are rapidly killed by phagocytic cells (Johnston, 1991).

It will be evident that a myriad of cells and molecules of the immune system cooperate to protect the lung, and that the interplay between host defences and pneumococcal factors is complex. Failure of these defences to clear inhaled bacteria from the lower respiratory tract is a balance between the effectiveness of host defences, the size and nature of the inoculum and the virulence potential of the pneumococcus in question. Pneumococcal multiplication in the lower respiratory tract gives rise to the classical features of lobar pneumonia, which proceeds in an overlapping series of steps (Johnston,

1991; Roberts, 1985). Initially, there is alveolar engorgement associated with oedema and bacteria in the alveoli. Red hepatization follows PMNL infiltration and the appearance of blood-tinged fluid: bacteria, fibrin and erythrocytes abound in the alveoli. Grey hepatization is apparent as the alveoli become progressively blocked by fibrin and PMNLs, and bacteria are cleared. Resolution accompanies the influx of macrophages and following recovery there is little evidence of residual tissue damage. A variety of pneumococcal factors may play a key role in the induction of the inflammatory response, and it has been shown that cell wall components can induce pulmonary inflammation (Tuomanen *et al.*, 1987) and pneumolysin (see below) alone can produce all the salient histological features of pneumonia in experimental animals.

Bacteraemia/septicaemia frequently accompanies lower respiratory tract infection but septicaemia in the absence of a primary focus of infection can occur. The site at which pneumococci gain access to the bloodstream and the mechanism by which this occurs is not clear, although several routes are possible which need not be mutually exclusive. In some studies, pneumococci are found in the blood only a few minutes after intranasal challenge of mice and might have invaded via the nasopharyngeal mucosa (Rake, 1936). Invasion from the lower respiratory tract, perhaps involving transport in the lymphatics via the hilar nodes, is also possible since pneumococci were also found in alveoli shortly after challenge. Similar experiments in rabbits (Schulz *et al.*, 1938) indicated that bacteraemia was preceded by the appearance of pneumococci in the cervical lymphatics. In intravenously challenged rabbits, pneumococci can cross both vascular and lymphatic endothelium and the lymphatics can serve as a reservoir of replicating pneumococci even in the presence of anti-pneumococcal antibody (Field *et al.*, 1937). Additionally, pneumococci in the subarachnoid space (see below) may also gain access to the cerebral venous system (Austrian, 1982*b*).

Both the liver and the spleen in conjunction with antibody play an important role in the clearance of pneumococci from the bloodstream (Austrian, 1982*b*). The spleen is thought to be important in the synthesis of antibody, in bacterial clearance in the non-immune host and to play a role in alternative pathway activation (Wara, 1982). Hence it is not surprising that the splenectomized individual is at particular risk from pneumococcal infection (Wara, 1982). The acute-phase reactant C-reactive protein (CRP) may also facilitate elimination of pneumococci from the blood (Briles *et al.*, 1989; Horowitz *et al.*, 1987). CRP is an acute-phase protein, first identified by its ability to bind to the C-polysaccharide from the pneumococcal cell wall. Following binding to its ligand, CRP activates the classical

pathway of complement via direct binding of the C1q component of complement to ligand-fixed CRP (Volanakis & Kaplan, 1974). In this regard CRP acts as a primitive antibody and as such may be an important component of non-specific defence against bacterial infection in the preimmune phase of infection. Indeed, passive immunisation of mice with human CRP was found to protect mice from pneumococcal challenge (Mold *et al.*, 1981; Yother *et al.*, 1982).

The presence of pneumococci in the blood of man and animals with pneumonia correlates with poor prognosis although the reasons why infected individuals die from the infection is still not known. The 'toxic' nature of pneumococcal infections has been highlighted on many occasions (Johnston, 1991), although the specific nature of the pneumococcal component responsible for the observed toxicity has not been definitively identified. The polysaccharide capsule, although required for *in vivo* multiplication, is not toxic and thus other pneumococcal factors must be important (either directly or indirectly) in promoting damage. The elimination of pneumococci from infected individuals using antibiotics often fails to alleviate the fatal outcome of infection. Dick & Gemmell (1971) were able to reproduce this effect in infected mice. These authors interpret this to indicate that a pneumococcal toxin, perhaps pneumolysin (see below), plays a part in the fatal outcome of the infection. The observation that pneumolysin antibodies delay the fatal outcome of pneumococcal infection in mice (Paton *et al.*, 1983) might lend credence to this suggestion. Alternatively, it is possible that the bacterial load is sufficiently high that the induction of pneumococcal lysis by the antibiotic releases a variety of pneumococcal products which induce irreversible shock. Elevated serum levels of tumour necrosis factor (TNF) (Nohynk *et al.*, 1991) and raised levels of interleukin-1 in broncho-alveolar washings (Wilmott *et al.*, 1990) have been observed in children with acute lower respiratory tract infections. Interestingly, pneumococcal cell wall components have been reported to stimulate interleukin-1 but not TNF production from human monocytes (Riesenfeld-orn *et al.*, 1989). The patterns of cytokine production induced by the action of pneumolysin on immune cells is not known.

The mechanisms and route by which pneumococci gain access to the meninges and cause meningitis is not clear. Haematogenous spread is one possibility, although meningitis rarely ensues following intravenous inoculation of animals with pneumococci (Moxon, 1982). It was observed that pneumococci rapidly appeared in the subarachnoid space following intranasal challenge of mice and it was speculated that they did so via the olfactory mucosa (Rake, 1937), perhaps involving the perineural spaces around olfactory neurons (Moxon,

1982). Pneumococcal neuraminidase has been implicated in the pathogenesis of meningitis (see below).

Pneumococcal virulence factors

The pneumococcus produces a number of products having properties consistent with a role in promotion and maintenance of the carrier state and the pathogenesis of disease. However only in the cases of the capsular polysaccharide (Austrian, 1982*a*), pneumococcal surface protein A, and the toxin pneumolysin (Berry *et al.*, 1989*a*) is there clear evidence that they are virulence factors. Autolysin may also contribute to the disease process (see below) although this may be an indirect effect consequent on autolysin-mediated release of pneumococcal cell wall and other factors (Berry *et al.*, 1989*a*).

Capsule

The capsule of the pneumococcus plays a key role in virulence since non-capsulate derivatives of previously virulent pneumococci show a reduction in virulence of several orders of magnitude and the capsule acts to restrict opsonophagocytosis of the bacterium (Austrian, 1982*b*). Some 83 capsular polysaccharides have been described on the surface of the pneumococcus and these form the basis of the serological typing of *S. pneumoniae*. The association of capsular type and disease is well documented, as is the importance of anticapsular antibodies in protection against pneumococcal infection. These observations have led to the introduction of a 23-valent polysaccharide vaccine against pneumococcal infection; whilst this has proved effective in protecting at-risk adults it is of questionable efficacy in those of the population most at risk from the disease (van Dam *et al.*, 1990). This reflects the fact that capsular polysaccharides are often weak immunogens, eliciting poor antibody responses, and because of the T-cell independence of the response fail to induce memory.

Surface protein

Pneumococcal surface protein A (PspA) is present on the surface of many, if not all, clinical isolates of the pneumococcus (Crain *et al.*, 1990) and has a postulated structure which is unique, although some similarities to other surface proteins in Gram-positive bacteria (e.g. M proteins in group A streptococci) are evident (Yother *et al.*, 1991). Passive immunization with monoclonal antibodies raised against PspA can protect mice against intravenous challenge with certain pneumococcal strains (Briles *et al.*, 1989; McDaniel *et al.*, 1984, 1986). Despite

the extensive serological variation in PspA, recombinant PspA from one pneumococcal serotype was shown to confer protection in mice to challenge with different pneumococcal serotypes (McDaniel *et al.*, 1991). The *pspA* gene has been inactivated in a number of virulent pneumococci (McDaniel *et al.*, 1987), resulting in an attenuation of virulence, and in some cases the strains were rendered avirulent (Briles *et al.*, 1988). During the first hour following challenge these PspA⁻ pneumococci were more rapidly cleared from mice than their wild-type parents, although after this time there was no obvious difference in clearance or growth rates. Whilst all animals challenged with these mutant pneumococci died from the infection, they did so later than those infected with the wild-type strain (Briles *et al.*, 1988). In this regard the phenotype of the PspA⁻ and pneumolysin-negative pneumococci (see below) were similar.

Neuraminidase

All pneumococcal isolates produce neuraminidase (Kelly *et al.*, 1967) and although this enzyme may facilitate pneumococcal colonization (see above), it has also been implicated in the pathogenesis of meningitis since intracerebral inoculation of the enzyme in mice induces neurological signs (Kelly & Greiff, 1970). However, these experiments involved only crude preparations of the enzyme and contamination of these preparations with pneumococcal cell wall material would have produced the same result since cell wall components alone have been shown to induce meningeal inflammation (Tuomanen *et al.*, 1985). Nevertheless the observation that immunization of mice with purified neuraminidase conferred limited protection against challenge with virulent pneumococci may indicate that neuraminidase is a pneumococcal virulence factor (Lock *et al.*, 1988).

The number of neuraminidases produced by the pneumococcus has been the subject of controversy. There are reports of several pneumococcal neuraminidase isoenzymes (Tanenbaum *et al.*, 1970; Tanenbaum & Sun, 1971) or the existence of a single neuraminidase which is highly prone to proteolytic degradation (Berry *et al.*, 1988). Recently the genes for two pneumococcal neuraminidases have been cloned (Berry *et al.*, 1988; Camara *et al.*, 1991) and shown to be distinct, and both genes were found in individual pneumococcal isolates of different serotypes (Camara *et al.*, 1991). The relationship between the products of these genes in terms of structure and substrate specificity is not clear and an understanding of these points is clearly important if the role (if any) of neuraminidase in pneumococcal disease is to be elucidated. In an attempt to ascertain the role neuraminidases play in disease we are currently isolating neuraminidase-negative mutants of the pneumococcus.

Hyaluronidase

Pneumococci and other streptococci produce hyaluronidase (Humphrey, 1948), an enzyme which depolymerizes hyaluronic acid. The degradation of this important component of the extracellular matrix has been postulated to contribute to the spread of certain streptococci through tissue and it is tempting to hypothesize that the enzyme might contribute to the invasive potential of the pneumococcus. We have recently cloned the gene for pneumococcal hyaluronidase (Boulnois *et al.*, 1991*b*; A. Shepherd and others, unpublished data) as a first step towards the testing of this hypothesis.

Pneumolysin

Pneumolysin is produced by all isolates of the pneumococcus, and the cloning and sequencing of two pneumolysin genes from different serotypes obtained from disparate geographical regions and isolated several decades apart revealed that the proteins differed by only a single amino acid (Mitchell *et al.*, 1990*a*).

There is good evidence to indicate that pneumolysin is produced during natural infections of man and experimental infections in animals. Antibody titres against pneumolysin have been reported to increase during pneumococcal infection of man (Jalonen *et al.*, 1989; Kanclerski *et al.*, 1988), indicating that the toxin is produced *in vivo*. In addition, in preliminary experiments, we have been able to detect pneumolysin directly in lung, spleen and liver tissue taken from mice challenged intranasally with pneumococci (T. J. Mitchell and others, unpublished data).

Several lines of evidence indicate that pneumolysin contributes directly to the virulence potential of the pneumococcus. Immunization of mice with native (Paton *et al.*, 1983) and recombinant (Paton *et al.*, 1991) pneumolysin conferred limited protection against an intranasal challenge with virulent pneumococci in a serotype-independent fashion. Although the majority of immunized animals eventually succumbed to the infection, they survived longer than their unimmunized counterparts. Interestingly, when animals immunized with pneumolysin inactivated by site-directed mutagenesis were challenged via the intraperitoneal route, the majority of them survived (Paton *et al.*, 1991). A pneumolysin-negative pneumococcus, generated by insertion-duplication mutagenesis, had reduced virulence for mice and was more rapidly cleared from the bloodstream than the wild-type strain (Berry *et al.*, 1989*b*). As observed with PspA⁻ pneumococci, mice infected with pneumolysin-negative mutants died much later than the equivalent animals infected with the isogenic wild-type parent. This delay in the fatal outcome

of the infection was similar to that observed with the pneumolysin-immunized animals (see above). Thus pneumolysin might be another pneumococcal factor which facilitates *in vivo* multiplication, and the toxin's ability to interfere with phagocyte and immune cell function may be important in this regard (see below). However, the effects of pneumolysin on host tissue are likely to be diverse given that all mammalian cells are, to a greater or lesser extent, susceptible to the toxin. For example, pneumolysin inhibits the beating of cilia on human respiratory tract epithelium (Feldman *et al.*, 1990; Steinfors *et al.*, 1989) and, since beating of cilia is an important facet of the non-specific defences of the respiratory tract, this may promote appearance of pneumococci in the lower respiratory tract.

Pneumolysin belongs to a family of toxins known as the thiol-activated toxins, because they lose activity on oxidation but regain full activity following addition of reducing agents (Smyth & Duncan, 1978). Thiol-activated toxins are produced by four genera of Gram-positive bacteria and have an assumed common mode of action on mammalian cells. The presence of cholesterol in membranes appears to be a critical determinant of the susceptibility of cells to these toxins and free cholesterol is a potent inhibitor of cytolytic activity (Smyth & Duncan, 1978). The generally accepted mode of cytolytic action (Alouf, 1980; Bhakdi & Tranum-Jensen, 1987; Bhakdi & Tranum-Jensen, 1988; Smyth & Duncan, 1978) involves the binding of monomeric toxin to mammalian cells via an interaction with cholesterol and the temperature-dependent insertion of the protein into the lipid bilayer. Aggregation of membrane-fixed toxin results in the generation of cholesterol-free toxin oligomers containing up to 80 monomers. These are thought to correspond to the ring and arc structures seen in treated cell membranes, which correlate with the appearance of transmembrane pores some 30–35 nm in diameter.

As well as causing cytolysis of mammalian cells, some thiol-activated toxins are cytotoxic to phagocytes and cells of the human immune system at concentrations (2 ng ml⁻¹) which have no effect on cell viability (Ferrante *et al.*, 1984; Paton & Ferrante, 1983; Van Epps & Andersen, 1974). Pneumolysin inhibits neutrophil bactericidal activity (Paton & Ferrante, 1983) (by reducing chemotaxis, phagocytosis and the respiratory burst) and inhibits lymphocyte proliferation in response to mitogens and the synthesis of all immunoglobulin classes (Ferrante *et al.*, 1984). These aspects of host defence are known to be important in protection against pneumococcal disease.

Although thiol-activated toxins are generally considered to be hydrophobic proteins (Johnson *et al.*, 1982), the predicted sequences of five members of this toxin

family [pneumolysin, streptolysin O (SLO), listeriolysin, perfringolysin and alveolysin] that are available contain no extended stretches of hydrophobicity (Geoffroy *et al.*, 1990; Kehoe *et al.*, 1987; Kehoe & Timmis, 1984; Mengaud *et al.*, 1988; Tweten, 1988; Walker *et al.*, 1987). Thus the hydrophobic faces of these molecules which interact with membranes may be generated by the conformation they adopt in the membrane. The sequence of pneumolysin (Walker *et al.*, 1987) lacks an N-terminal signal sequence, consistent with the reported cytoplasmic location of the protein in the pneumococcus (Johnson, 1977). The thiol-activated toxins show serological cross-reactivity (Smyth & Duncan, 1978) and there is extensive primary amino acid sequence homology between them (Geoffroy *et al.*, 1990; Kehoe *et al.*, 1987; Mengaud *et al.*, 1988; Tweten, 1988; Walker *et al.*, 1987).

The formation and breakage of intra-molecular disulphide bonds was previously thought to be the basis of the observed thiol activation, an hypothesis which implied that each toxin monomer contained at least two cysteine residues. Amino acid composition analysis of several thiol-activated toxins yielded varying estimates for their cysteine content (Geoffroy *et al.*, 1981; Smyth & Duncan, 1978). However, for each of the toxins sequenced only one cysteine residue per monomer was found and this lies towards the C-terminus of the proteins in a conserved eleven-amino-acid sequence (-ECTGLAWWW-). This is inconsistent with thiol activation involving the formation and breakage of intramolecular disulphide bridges (Alouf, 1980; Smyth & Duncan, 1978).

The findings that oxidized toxin was not cytolytic and had lost the ability to bind to cells, and that SLO and alveolysin when treated with thiol-blocking agents lost cytolytic activity, suggested a critical role for a single thiol group in membrane-damaging activity (Alouf, 1980; Bhakdi & Trantum-Jensen, 1988; Geoffroy *et al.*, 1981; Smyth & Duncan, 1978). This essential thiol group was postulated to have a role in cholesterol binding, direct induction of membrane damage or maintenance of an active conformation (Alouf, 1980; Bhakdi & Trantum-Jensen, 1988; Smyth & Duncan, 1978). The role of the unique cysteine in pneumolysin was probed using site-directed mutagenesis to change the single cysteine to alanine and, surprisingly, the modified toxin retained full cytolytic and cytotoxic activity (Saunders *et al.*, 1990). Similar observations have been made with SLO (Pinkney *et al.*, 1989) and listeriolysin (Michel *et al.*, 1990). Thus there is no obvious role for the thiol group in the *in vitro* activity of pneumolysin, although it remains possible that the thiol group is important *in vivo*. For example, it may stabilize the toxins *in vivo* by promoting an interaction with other thiol-containing proteins.

Site-directed mutagenesis studies have revealed that

the cysteine-containing motif is important for cytolytic and cytotoxic activity. When the unique cysteine in pneumolysin was changed to either serine or glycine, the cytolytic activity of the toxin was reduced, but not abolished (Saunders *et al.*, 1990). The reduced activity of these toxins could not be wholly attributed to a defect in cell binding or an inability to form membrane-associated oligomers (Saunders *et al.*, 1990). A similar result was obtained when tryptophans at positions 433 and 435 in pneumolysin were changed independently to phenylalanine (Boulnois *et al.*, 1990). Similar findings were made with SLO (Pinkney *et al.*, 1989) and listeriolysin (Michel *et al.*, 1990). Thus the cysteine-containing motif appears to participate in the generation of the functional lesion in toxin-treated membranes after binding and oligomerization. We have recently observed that many of these modified pneumolysin molecules had a reduced, but still detectable, capacity to promote leakage of phosphorylcholine from mammalian cells (C. Pasternak and others, unpublished data), pointing to a role for the cysteine-containing motif in pore formation. In addition, these mutant toxins were found to have a reduced susceptibility to cation inhibition compared with the wild-type toxin (C. Pasternak and others, unpublished data).

Despite the conservation of the cysteine-containing motif, specific residues are not required for activity, suggesting that the overall structure of the motif is important. We postulated that this sequence motif promotes an interaction with cholesterol that is required for pore formation. We suggested this on the basis that a similar group of tryptophan residues is found in cholesterol oxidase (Boulnois *et al.*, 1990). Additionally, tryptophan residues towards the C-terminus of the channel-forming polypeptide antibiotic gramicidin A are thought to play an essential role in channel formation by promoting an interaction with the ring structures of the sterol to orientate the polypeptide in the membrane so that hydrophilic domains span the membrane resulting in the pore formation (de Kruijff, 1990). This sequence of events may also apply to pneumolysin and the other thiol-activated toxins (de Kruijff, 1990).

Treatment of pneumolysin with the histidine-modifying agent diethyl pyrocarbonate (DEPC) abolished cytolytic activity and prevented toxin binding to cells (Boulnois *et al.*, 1990; T. J. Mitchell and others, unpublished data). Only one conserved histidine (His₃₆₇ in pneumolysin) is present in each of the toxins for which a predicted sequence is available and this residue was changed to arginine in pneumolysin. The modified toxin had only residual cytolytic activity but was shown to bind to cells normally yet was unable to form oligomers in membranes (Boulnois *et al.*, 1990; T. J. Mitchell and others, unpublished data). Thus His₃₆₇ in pneumolysin is involved in promoting either toxin-toxin or toxin-

membrane interactions required for oligomer formation. Histidine residues have also been implicated in the pentamerization or hexamerization of the toxin aerolysin (Green & Buckley, 1990). Interactions between cholesterol and the thiol-activated toxins are also thought to be important in oligomerization (Smyth & Duncan, 1978). The formation of functional pneumolysin oligomers may involve the cooperative action of His₃₆₇ and the cysteine-containing motif.

The mechanism involved in the binding of toxin to mammalian cells is not understood, although in most models cholesterol is assumed to act as receptor (Alouf, 1980; Bhakdi & Trantum-Jensen, 1988; Smyth & Duncan, 1978). However, the evidence for this is only circumstantial and the relative inaccessibility of cholesterol within the membrane (Yeagle, 1987) may indicate a receptor distinct from the sterol. Toxin binding to cholesterol in cell membranes was proposed to involve a discrete region in the protein, the fixation site, since neutralizing antibodies which blocked SLO binding had no effect on activity of toxin pre-bound to cells (Alouf, 1980). Membrane binding has been postulated also to involve a hydrophobic interaction between toxin and sterol, or hydrogen bonding between several amino acids in the toxin and the 3 β -OH groups of cholesterol (Alouf, 1980; Smyth & Duncan, 1978). The work discussed above suggests that toxin-receptor interactions do not involve the cysteine motif. In agreement with this notion, an N-terminal fragment of listeriolysin, lacking the cysteine-containing motif, bound to free cholesterol (Vazquez-Boland *et al.*, 1989). In contrast, the cysteine in perfringolysin was proposed to mediate both receptor binding and membrane damage (Iwamoto *et al.*, 1987). These apparently conflicting conclusions may indicate that free and membrane-associated cholesterol interact with different regions of the toxins. Alternatively, these conflicting views may reflect experimental difficulties associated with the use of cholesterol in aqueous solution.

Pneumolysin can activate the classical pathway of complement in the absence of anti-toxin antibodies (Paton *et al.*, 1984). This may be of some consequence since complement is an important component of host defence against the pneumococcus (Winkelstein, 1982, 1984). In the fluid phase the ability of pneumolysin to activate complement may divert complement from the invading pneumococci and hence compromise complement-mediated opsonophagocytosis. If activation occurs on membrane-fixed toxin, as has been shown for SLO (Bhakdi & Trantum-Jensen, 1985), then this may result in the assembly of the membrane attack complex of complement on autologous membranes, which may contribute to tissue damage. Both scenarios would be expected to stimulate inflammatory responses (Bhakdi & Trantum-Jensen, 1985).

During the development of a pneumolysin-based ELISA, we observed a non-immune reaction between pneumolysin and antibody (Boulnois *et al.*, 1990, 1991*a*; Mitchell *et al.*, 1991). We subsequently demonstrated that intact antibody and purified Fc (but not Fab) fragments of antibody were bound by the toxin. Thus pneumolysin is another example of a bacterial protein with Fc-binding capacity. However we were unable to detect sequence homology between pneumolysin and other Fc-binding proteins. Pneumolysin lacks the antibody-binding repeat sequences that are typical of other Fc-binding proteins. However, it seemed reasonable to suppose that antibody binding and complement activation by pneumolysin might be related.

A clue to the domains of pneumolysin involved in complement activation was obtained when we observed that pneumolysin (and other thiol-activated toxins) has limited sequence homology with human CRP (Boulnois *et al.*, 1990, 1991*b*; Mitchell *et al.*, 1990*b*, 1991). A C-terminal sequence within CRP has homology with two non-contiguous segments in pneumolysin. CRP activates the classical pathway of complement in the absence of antibody via direct binding of the C1q component of complement to ligand-fixed CRP (Volanakis & Kaplan, 1974). It was thus intriguing that this bacterial protein toxin and human CRP share homology.

Site-directed mutagenesis of both CRP-like regions of pneumolysin has been carried out (Boulnois *et al.*, 1990, 1991*b*; Mitchell *et al.*, 1990*b*, 1991). Of the changes made in region 1 of pneumolysin (Phe₂₆₃ to Trp, Glu₂₆₄ to Gln, Glu₂₇₇ to Gln and Trp₂₇₈ to Phe), none affected complement activation or antibody binding. In contrast, changes in region 2 (Trp₃₇₉ to Phe, Tyr₃₈₄ to Phe, Asp₃₈₅ to Asn and Trp₃₉₇ to Phe) reduced both properties. The Tyr₃₈₄ to Phe and the Asp₃₈₅ to Asn modified toxins had only background ability to activate complement and an antibody-binding capacity reduced by about 70%. Thus we postulated that complement activation and antibody binding are interrelated and that non-immune binding of antibody contributes to classical pathway activation (Boulnois *et al.*, 1990, 1991*a*; Mitchell *et al.*, 1990*b*, 1991). Whether or not the homologous sequence in CRP also promotes antibody binding is not known but CRP-mediated complement activation requires only C1q. A preliminary experiment revealed that pneumolysin also can bind C1q directly, although changes made in the CRP-like region 2 of pneumolysin had no effect on C1q binding (T. J. Mitchell and others, unpublished data); however, it remains possible that CRP-like region 1 of pneumolysin is involved in C1q binding. Whether or not C1q binding by pneumolysin contributes to classical pathway activation is not known.

The mechanisms by which pneumolysin binds antibody via Fc and activates complement is not clear,

although Tyr₃₈₄ and Asp₃₈₅ appear to be important. A tyrosine–acidic amino acid pair is also found in the IgG-binding domains of proteins A and G and, in the former, the tyrosine residue is known to interact with antibody (Fahnestock *et al.*, 1990; Guss *et al.*, 1990). More than one molecule of IgG in close proximity is normally required to activate the classical pathway of complement (Wright *et al.*, 1980). This might be achieved indirectly by pneumolysin as a consequence of its tendency to aggregate in solution, such that monovalent binding of antibody by pneumolysin would suffice to activate complement. Alternatively, pneumolysin might have more than one antibody-binding site, and it is interesting that the Asp₃₈₅ to Asn modified pneumolysin retains some capacity to bind antibody despite being unable to activate complement. Other models for complement activation by pneumolysin require that C1q binding by the toxin contributes to activation. For example, the globular heads of C1q might bind directly to the toxin and the Fc domain of antibody bound to CRP-like region 2. Alternatively, the Fc domain of a single antibody might bring two molecules of toxin, and hence two C1q binding sites, into close apposition.

Whether or not complement activation by pneumolysin is important *in vivo* during normal infection with the pneumococcus is not known. Pneumolysin might act as a CRP analogue but without the appropriate ligand-binding properties. In this way pneumolysin might compete with CRP and abrogate its protective effect. Purified recombinant pneumolysin, when injected into the ligated lobe of a rat lung, causes the same histological features of lobar pneumonia as does inoculation of similar lobes with virulent pneumococci (Feldman *et al.*, 1991). Administration of modified pneumolysin defective in either membrane-damaging capacity or complement activation caused less inflammation, providing evidence that pneumolysin-mediated complement activation can occur *in vivo* and thus has the potential to contribute to disease. Pneumolysin may act in conjunction with cell wall components (Tuomanen *et al.*, 1987) to induce inflammation in the lung.

Autolysin and the release of pneumococcal virulence factors

It is clear that a number of pneumococcal factors are potentially important in the pathogenesis of disease. However, in the case of pneumolysin (Johnson, 1977) and perhaps neuraminidase (Lock *et al.*, 1988), these factors appear to be located in the cytoplasm of the pneumococcus. Both of these proteins are released from the cell during autolysis, a process mediated by the cell-wall-associated autolysin. Interestingly, a defined autoly-

sin-negative mutant of the pneumococcus had reduced virulence in mice following intranasal challenge and immunization of mice with autolysin conferred limited protection against intranasal challenge with wild-type pneumococci (Berry *et al.*, 1989a). Thus autolysin contributes to virulence, although this may be indirect, with the enzyme releasing proteins and cell wall fragments which can contribute to virulence and inflammation.

An improved pneumococcal vaccine?

The above discussion serves to highlight the potential importance of pneumolysin in the pathogenesis of disease, and the ability of the toxin to confer limited protection upon mice in a serotype-independent manner has been described (Paton *et al.*, 1983, 1991). The current pneumococcal vaccine, comprising 23 polysaccharides from the most frequently encountered pneumococcal serotypes, has significant shortcomings. These relate primarily to the poor immunogenicity of capsular polysaccharides but many studies have shown that immunogenicity and memory to polysaccharide antigens can be achieved by the conjugation of the polymer to a protein carrier. Pneumolysin may be the ideal carrier for the pneumococcal polysaccharide vaccine since improved immunogenicity may accompany induction of anti-toxin antibodies which themselves may be protective in a serotype-independent way. The genetically toxoided pneumolysin molecules described above would be ideal for this purpose since they have dramatically reduced biological activities. We have recently reported the purification and immunogenicity of the toxoided derivatives of pneumolysin to the type 19F polysaccharide (Paton *et al.*, 1991). The 19F polysaccharide was chosen because it is a particularly poor immunogen in children and mice. The conjugation was found to convert the 19F polymer into an antigen capable of inducing a booster effect and it seems likely that coupling the 19F polysaccharide to pneumolysin converted it into a T-cell-dependent antigen. The conjugate also gave modest responses to pneumolysin and it will be interesting to assess if any protection afforded is serotype independent.

This work was supported in part by grants from the MRC and the Wellcome Trust. G.J.B. is a Lister–Jenner Fellow. I am indebted to many collaborators (Dr J. Paton, Dr R. Wilson, Dr F. Mendez, Professor C. Pasternak, Dr L. Bashford) who made this work possible. I am particularly pleased to thank my colleagues, Dr Peter Andrew and Dr Tim Mitchell, of the Microbiology Department at Leicester, for their longstanding collaboration and help in ways too numerous to mention. A number of PhD students (J. Walker, S. Pratt, A. Shepherd, K. Saunders, J. Canvin, R. Owen and M. Camara-Garcia) have also played a central role in these studies.

References

- ALOUF, J. E. (1980). Streptococcal toxins (streptolysin O, Streptolysin S, erythrogenic toxin). *Pharmacology and Therapeutics* **11**, 661–717.
- ANDERSSON, B., ERIKSSON, B., FALSEN, E., FOGH, A., HANSON, L. A., NYLEN, O., PETERSON, H. & SVANBORG-EDEN, C. (1981). Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells *in vitro*: differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. *Infection and Immunity* **32**, 311–317.
- ANDERSSON, B., DAHMEN, J., FREJD, T., LEFFLER, H., MAGNUSSON, G., NOORI, G. & EDEN, C. S. (1983). Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human epithelial cells. *Journal of Experimental Medicine* **158**, 559–570.
- ANDERSSON, B., BEACHEY, E. H., TOMASZ, A., TUOMANEN, E. & SVANBORG-EDEN, C. (1988). A sandwich adhesin on *Streptococcus pneumoniae* attaching to oropharyngeal epithelial cells *in vitro*. *Microbial Pathogenesis* **4**, 267–268.
- AUSTRIAN, R. (1982a). Pneumococcus: the first one hundred years. In *The Pneumococcus and the Pneumococcus Vaccine*, pp. 1–7. Edited by P. G. Quie & E. H. Kass. Chicago: University of Chicago Press.
- AUSTRIAN, R. (1982b). Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. In *The Pneumococcus and the Pneumococcal Vaccine*, pp. 191–207. Edited by P. G. Quie & E. H. Kass. Chicago: University of Chicago Press.
- AUSTRIAN, R. (1986). Some aspects of the pneumococcal carrier state. *Journal of Antimicrobial Chemotherapy* **18**, 35–45.
- AUSTRIAN, R., HOWIE, V. M. & PLOUSSARD, J. H. (1977). The bacteriology of pneumococcal otitis media. *Johns Hopkins Medical Journal* **141**, 104–111.
- BERRY, A. M., PATON, J. C., GLARE, D., HANSMAN, D. & CATCHESIDE, D. E. A. (1988). Cloning and expression of the pneumococcal neuraminidase in *Escherichia coli*. *Gene* **71**, 199–305.
- BERRY, A. M., LOCK, R. A., HANSMAN, D. & PATON, J. C. (1989a). Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infection and Immunity* **57**, 2324–2330.
- BERRY, A. M., YOTHER, J., BRILES, D. E., HANSMAN, D. & PATON, J. C. (1989b). Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. *Infection and Immunity* **57**, 2037–2042.
- BHAKDI, S. & TRANUM-JENSEN, J. (1985). Complement activation and attack on autologous cells induced by streptolysin-O. *Infection and Immunity* **48**, 713–719.
- BHAKDI, S. & TRANUM-JENSEN, J. (1987). Damage to mammalian cells by proteins that form transmembrane pores. *Reviews of Physiology, Biochemistry and Pharmacology* **107**, 147–223.
- BHAKDI, S. & TRANUM-JENSEN, J. (1988). Damage to cell membranes by pore-forming bacterial cytolysins. *Progress in Allergy* **40**, 1–43.
- BOULNOIS, G. J., MITCHELL, T. J., SAUNDERS, F. K., MENDEZ, F.-J. & ANDREW, P. W. (1990). Structure and function of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. In *Bacterial Protein Toxins*, pp. 43–51. Edited by R. Rappuoli and others. Stuttgart: Gustav Fischer.
- BOULNOIS, G. J., MITCHELL, T. J., SAUNDERS, K., OWEN, R., CANVIN, J., SHEPHERD, A., CAMARA, M., WILSON, R., FELDMAN, C., STEINFORT, C., BASHFORD, L., PASTERNAK, C. & ANDREW, P. W. (1991a). Analysis of some putative protein virulence factors of *Streptococcus pneumoniae*. In *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*, pp. 83–87. Edited by G. M. Dunny, P. P. Cleary & L. L. McKay. Washington, DC: American Society for Microbiology.
- BOULNOIS, G. J., MITCHELL, T. J., PATON, J. C. & ANDREW, P. W. (1991b). Structure and function of pneumolysin, the multifunctional, membrane damaging toxin of *Streptococcus pneumoniae*. *Molecular Microbiology* **5**, 2611–2616.
- BRILES, D. E., YOTHER, J. & MCDANIEL, L. S. (1988). Role of pneumococcal surface protein A in the virulence of *Streptococcus pneumoniae*. *Reviews of Infectious Diseases* **10**, S372–S374.
- BRILES, D. E., FORMAN, C., HOROWITZ, J. C., VOLANAKIS, J. E., BENJAMIN, J. W. H., MCDANIEL, L. S., ELDRIDGE, J. & BROOKS, J. (1989). Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. *Infection and Immunity* **57**, 1457–1464.
- BUSSE, W. (1991). Pathogenesis and sequelae of respiratory infections. *Reviews of Infectious Diseases* **13**, S477–S485.
- CAMARA, M., MITCHELL, T. J., ANDREW, P. W. & BOULNOIS, G. J. (1991). *Streptococcus pneumoniae* produces at least two distinct enzymes with neuraminidase activity: cloning and expression of a second neuraminidase gene in *Escherichia coli*. *Infection and Immunity* **59**, 2856–2858.
- CRAIN, M. J., WALTMAN, W. D., II, TURNER, J. S., YOTHER, J., TALKINGTON, D. F., MCDANIEL, L. S., GRAY, B. M. & BRILES, D. E. (1990). Pneumococcal surface protein-A (PspA) is serologically highly variable and is expressed by all clinically important serotypes of *Streptococcus pneumoniae*. *Infection and Immunity* **58**, 3293–3299.
- VAN DAM, J. E. G., FLEER, A. & SNIPPE, H. (1990). Immunogenicity and immunochemistry of *Streptococcus pneumoniae* capsular polysaccharides. *Antonie van Leeuwenhoek* **58**, 1–47.
- DICK, T. B. & GEMMELL, C. G. (1971). The pathogenesis of pneumococcal infection in mice. *Journal of Medical Microbiology* **4**, 153–163.
- FAHNESTOCK, S. R., ALEXANDER, P., FILPULA, D. & NAGLE, J. (1990). Structure and evolution of the streptococcal genes encoding protein G. In *Bacterial Immunoglobulin Binding Proteins*, pp. 133–148. Edited by M. D. P. Boyle. London: Academic Press.
- FELDMAN, C., MITCHELL, T. J., ANDREW, P. W., BOULNOIS, G. J., READ, R. C., TODD, H. C., COLE, P. J. & WILSON, R. (1990). The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium *in vitro*. *Microbial Pathogenesis* **9**, 275–284.
- FELDMAN, C., MUNRO, N. C., JEFFREY, R. K., MITCHELL, T. J., ANDREW, P. W., BOULNOIS, G. J., GUERREIRO, D., ROHDE, J. A. L., TODD, H. C., COLE, P. J. & WILSON, R. (1991). Pneumolysin induces the salient histological features of pneumococcal infection in the rat lung *in vivo*. *American Journal of Respiratory Cell and Molecular Biology* **5**, 416–423.
- FERRANTE, A., ROWAN-KELLY, B. & PATON, J. C. (1984). Inhibition of *in vitro* human lymphocyte response by the pneumococcal toxin pneumolysin. *Infection and Immunity* **46**, 585–589.
- FIELD, M. E., SHAFFER, M. F., ENDERS, J. F. & DRINKER, C. K. (1937). The distribution in the blood and lymph of pneumococcus type III injected intravenously in rabbits, and the effect of treatment with specific antiserum on the infection in lymph. *Journal of Experimental Medicine* **65**, 469–485.
- GEOFFROY, C., GILLES, A.-M. & ALOUF, J. E. (1981). The sulfhydryl groups of the thiol-dependent cytolytic toxin from *Bacillus alvei*: evidence for one essential sulfhydryl group. *Biochemical and Biophysical Research Communications* **99**, 781–788.
- GEOFFROY, C., MENGAUD, J., ALOUF, J. E. & COSSART, P. (1990). Alveolysin, the thiol-activated toxin of *Bacillus alvei*, is homologous to listeriolysin O, perfringolysin O, pneumolysin and streptolysin O and contains a single cysteine. *Journal of Bacteriology* **172**, 7301–7305.
- GREEN, M. J. & BUCKLEY, J. T. (1990). Site-directed mutagenesis of the hole-forming toxin aerolysin: Studies on the role of histidines in receptor binding and oligomerization of the monomer. *Biochemistry* **29**, 2177–2180.
- GREENWOOD, B. M. (1987). Pneumococcal infection. In *Oxford Textbook of Medicine*, pp. 5.182–5.191. Edited by D. J. Weatherall, J. G. G. Ledingham & D. A. Warrell. Oxford: Oxford University Press.
- GUSS, B., LINDBERG, M. & UHLEN, M. (1990). The gene for staphylococcal protein A. In *Bacterial Immunoglobulin Binding Proteins*, pp. 29–39. Edited by M. D. P. Boyle. London: Academic Press.
- GWALTNEY, G. S., SANDE, M. A., AUSTRIAN, R. & HENDLEY, J. O. (1975). Spread of *Streptococcus pneumoniae* in families. II. Relation of transfer of *S. pneumoniae* to incidence of colds and serum antibody. *Journal of Infectious Diseases* **132**, 62–68.
- HEFFRON, R. (1939). *Pneumonia with Special Reference to Pneumococcus Lobar Pneumonia*, p. 1086. New York: The Commonwealth Fund.
- HOROWITZ, J., VOLANAKIS, J. E. & BRILES, D. E. (1987). Blood clearance of *Streptococcus pneumoniae* by C-reactive protein. *Journal of Immunology* **138**, 2598–2603.

- HUMPHREY, J. H. (1948). Hyaluronidase production by pneumococci. *Journal of Pathology and Bacteriology* **55**, 273–275.
- IWAMOTO, M., OHNO-IWASHITA, Y. & ANDO, S. (1987). Role of the essential thiol group in the thiol-activated cytolysin from *Clostridium perfringens*. *European Journal of Biochemistry* **167**, 425–430.
- JALONEN, E., PATON, J. C., KOSKELA, M., KERTTULA, Y. & LEINONEN, M. (1989). Measurement of antibody responses to pneumolysin – a promising method for the presumptive aetiological diagnosis of pneumococcal pneumonia. *Journal of Infection* **19**, 127–134.
- JOHNSON, M. K. (1977). Cellular location of pneumolysin. *FEMS Microbiology Letters* **2**, 243–245.
- JOHNSON, M. K., KNIGHT, R. J. & DREW, G. K. (1982). The hydrophobic character of thiol-activated cytolysins. *Biochemical Journal* **207**, 557–560.
- JOHNSON, R. B. (1991). Pathogenesis of pneumococcal pneumonia. *Reviews of Infectious Diseases* **13**, S509–S517.
- KANCLERSKI, K., BLUMQUIST, S., GRANSTROM, M. & MOLLBY, R. (1988). Serum-antibodies to pneumolysin in patients with pneumonia. *Journal of Clinical Microbiology* **26**, 96–100.
- KEHOE, M. K. & TIMMIS, K. N. (1984). Cloning and expression in *Escherichia coli* of the streptolysin O determinant from *Streptococcus pyogenes*: characterization of the cloned streptolysin determinant and demonstration of the absence of substantial homology with determinants of other thiol-activated toxins. *Infection and Immunity* **43**, 804–810.
- KEHOE, M. K., MILLER, L., WALKER, J. A. & BOULNOIS, G. J. (1987). Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. *Infection and Immunity* **55**, 3228–3232.
- KELLY, R. & GREIFF, D. (1970). Toxicity of pneumococcal neuraminidase. *Infection and Immunity* **2**, 115–117.
- KELLY, R. T., FARMER, S. & GREIFF, D. (1967). Neuraminidase activity of clinical isolates of *Diplococcus pneumoniae*. *Journal of Bacteriology* **94**, 272–273.
- KILIAN, M. & REINHOLDT, J. (1986). Interference with IgA defence mechanisms by extracellular bacterial enzymes. In *Medical Microbiology*, pp. 173–208. Edited by C. S. F. Easmon & J. Jeljaszewicz. London: Academic Press.
- KRIVAN, H. C., ROBERTS, D. D. & GINSBERG, V. (1988). Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc- β 1-4-Gal found in some glycolipids. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 6157–6161.
- DE KRUIJFF, B. (1990). Cholesterol as a target for toxins. *Bioscience Reports* **10**, 127–130.
- LOCK, R. A., PATON, J. C. & HANSMAN, D. (1988). Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. *Microbial Pathogenesis* **5**, 461–467.
- MCDANIEL, L. S., SCOTT, G., KEARNEY, J. F. & BRILES, D. E. (1984). Monoclonal antibodies against protease sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. *Journal of Experimental Medicine* **160**, 386–397.
- MCDANIEL, L. S., SCOTT, G., WIDENHOFER, K., CARROLL, J. & BRILES, D. E. (1986). Analysis of a surface protein of *Streptococcus pneumoniae* recognised by protective monoclonal antibodies. *Microbial Pathogenesis* **1**, 519–531.
- MCDANIEL, L. S., YOTHER, J., VIJAYAKUMAR, M., MCGARRY, L., GUILD, W. R. & BRILES, D. E. (1987). Use of insertional activation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). *Journal of Experimental Medicine* **165**, 381–394.
- MCDANIEL, L. S., SHEFFIELD, J. S., DELUCCHI, P. & BRILES, D. E. (1991). PspA, a surface protein of *Streptococcus pneumoniae*, is capable of eliciting protection against pneumococci of more than one serotype. *Infection and Immunity* **59**, 222–228.
- MENGAUD, J., VINCENTE, M.-F., CHENEVERT, J., PEREIRA, J. M., GEOFFROY, C., GICQUEL-SANZEY, B., BAQUERO, F., PEREZ-DIAZ, J. C. & COSSART, P. (1988). Expression in *Escherichia coli* and sequence analysis of the lysteriolysin O determinant of *Listeria monocytogenes*. *Infection and Immunity* **56**, 766–772.
- MENGAUD, J., VINCENTE, M. F. V. & COSSART, P. (1989). Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes* *hlyA* region reveal structural features that may be involved in regulation. *Infection and Immunity* **57**, 3695–3701.
- MICHEL, E., REICH, K. A., FAVIER, R., BERCHE, P. & COSSART, P. (1990). Attenuated mutants of the intracellular bacterium *Listeria monocytogenes* obtained by single amino-acid substitutions in lysteriolysin O. *Molecular Microbiology* **4**, 2167–2178.
- MITCHELL, T. J., MENDEZ, F., PATON, J. C., ANDREW, P. W. & BOULNOIS, G. J. (1990a). Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2. *Nucleic Acids Research* **18**, 4010.
- MITCHELL, T. J., SAUNDERS, F. K., ANDREW, P. W. & BOULNOIS, G. J. (1990b). Complement activation by pneumolysin, location of domain and similarity to human acute phase protein. In *Bacterial Protein Toxins*, pp. 101–102. Edited by R. Rappuoli and others. Stuttgart: Gustav Fischer.
- MITCHELL, T. J., SAUNDERS, F. K., SMITH, A. N., ANDREW, P. W. & BOULNOIS, G. J. (1991). Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute phase protein. *Molecular Microbiology* **5**, 1883–1888.
- MOLD, C., NAKAYANA, S., HOLZER, T. J., GEWURZ, H. & DU CLOS, T. W. (1981). C-reactive protein is protective against *Streptococcus pneumoniae* in mice. *Journal of Experimental Medicine* **154**, 1703–1708.
- MOXON, E. R. (1982). Experimental infections of animals in the study of *Streptococcus pneumoniae*. In *The Pneumococcus and the Pneumococcal Vaccine*, pp. 172–175. Edited by P. G. Quie & E. H. Kass. Chicago: Chicago University Press.
- NOHYNK, H., TEPPA, A.-M., LAINE, E., LEINONEN, M. & ESKOLA, J. (1991). Serum tumor necrosis factor- α concentrations in children hospitalized for acute lower respiratory tract infection. *Journal of Infectious Diseases* **163**, 1029–1032.
- PATON, J. C. & FERRANTE, A. (1983). Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infection and Immunity* **41**, 1212–1216.
- PATON, J. C., LOCK, R. A. & HANSMAN, D. J. (1983). Effect of immunization with pneumolysin on the survival time of mice challenged *Streptococcus pneumoniae*. *Infection and Immunity* **40**, 548–552.
- PATON, J. C., ROWAN-KELLY, B. & FERRANTE, A. (1984). Activation of human complement by the pneumococcal toxin pneumolysin. *Infection and Immunity* **43**, 1085–1087.
- PATON, J. C., LOCK, R. A., LEE, C.-J., LI, J. P., BERRY, A. M., MITCHELL, T. J., ANDREW, P. W. & BOULNOIS, G. J. (1991). Purification and immunogenicity of genetically toxoided derivatives of pneumolysin and their conjugation to *Streptococcus pneumoniae* type 19F polysaccharide. *Infection and Immunity* **59**, 2297–2304.
- PINKNEY, M., BEACHEY, E. & KEHOE, M. (1989). The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infection and Immunity* **57**, 2553–2558.
- PLOTKOWSKI, M.-C., PUCHELLE, E., BECK, G., JACQUOT, J. & HANNOUN, C. (1986). Adherence of type 1 *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PR8 virus. *American Review of Respiratory Disease* **134**, 1040–1044.
- RAKE, G. (1936). Pathology of pneumococcus infection in mice following intranasal instillation. *Journal of Experimental Medicine* **63**, 17–31.
- RAKE, G. (1937). The rapid invasion of the body through the olfactory mucosa. *Journal of Experimental Medicine* **65**, 303–315.
- RIESENELD-ORN, I., WOLPE, S., GARCIA-BUSTOS, J. F., HOFFMANN, M. K. & TUOMANEN, E. (1989). Production of interleukin-1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. *Infection and Immunity* **57**, 1890–1893.
- RILEY, I. D. & DOUGLAS, R. M. (1982). An epidemiological approach to pneumococcal disease. In *The Pneumococcus and the Pneumococcal Vaccine*, pp. 51–63. Edited by P. G. Quie & E. H. Kass. Chicago: University of Chicago Press.
- ROBERTS, R. B. (1985). *Streptococcus pneumoniae*. In *Principles and Practices of Infectious Diseases*, pp. 1142–1152. Edited by G. L. Mandell, R. G. Douglas & J. E. Bennett. New York: Churchill Livingstone.

- SAUNDERS, F. K., MITCHELL, T. J., ANDREW, P. W. & BOULNOIS, G. J. (1990). Non-essential role for thiol groups in the *in vitro* activity of pneumolysin, the 'thiol-activated' toxin of *Streptococcus pneumoniae*. In *Bacterial Protein Toxins*, pp. 101–102. Edited by R. Rappuoli and others. Stuttgart: Gustav Fischer.
- SCHULZ, R. Z., WARREN, M. F. & DRINKER, C. K. (1938). The passage of rabbit virulent type III pneumococci from the respiratory tract of rabbits into the lymphatics and blood. *Journal of Experimental Medicine* **68**, 251–261.
- SMYTH, C. J. & DUNCAN, J. L. (1978). Thiol-activated (oxygen-labile) cytolyins. In *Bacterial Toxins and Cell Membranes*, pp. 129–183. Edited by J. Jeljaszewicz & T. Wadstrom. New York: Academic Press.
- STEINFORT, C., WILSON, R., MITCHELL, T., FELDMAN, C., RUTMAN, A., TODD, H., SYKES, D., WALKER, J., SAUNDERS, K., ANDREW, P. W., BOULNOIS, G. J. & COLE, P. J. (1989). Effect of *Streptococcus pneumoniae* on human respiratory epithelium *in vitro*. *Infection and Immunity* **57**, 2006–2013.
- TANENBAUM, S. W. & SUN, S. C. (1971). Some molecular properties of pneumococcal neuraminidase isoenzymes. *Biochimica et Biophysica Acta* **229**, 824–828.
- TANENBAUM, S. W., GULBINSKY, J., KATZ, M. & SUN, S. C. (1970). Separation, purification and some properties of pneumococcal neuraminidase isoenzymes. *Biochimica et Biophysica Acta* **198**, 242–254.
- TUOMANEN, E., LIU, H., HENGSTLER, B., ZAK, O. & TOMASZ, A. (1985). The induction of meningeal inflammation by components of the pneumococcal cell wall. *Journal of Infectious Diseases* **151**, 859–868.
- TUOMANEN, E., RICH, R. & ZAK, O. (1987). Induction of pulmonary inflammation by components of the pneumococcal cell wall. *American Review of Respiratory Disease* **135**, 869–874.
- TWETEN, R. K. (1988). Nucleotide sequence of the gene for perfringolysin O (theta-toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin O and pneumolysin. *Infection and Immunity* **56**, 3235–3240.
- VAN EPPS, D. E. & ANDERSEN, B. R. (1974). Streptolysin O inhibition of neutrophil chemotaxis and mobility: non-immune phenomenon with species specificity. *Infection and Immunity* **9**, 27–33.
- VAZQUEZ-BOLAND, J. A., DOMINGUEZ, L., RODRIGUEZ-FERRI, E. F., FERNANDEZ-GARAYZABAL, J. F. & SUAREZ, G. (1989). Preliminary evidence that different domains are involved in cytolytic activity and receptor (cholesterol) binding in listeriolysin O, the *Listeria monocytogenes* thiol-activated toxin. *FEMS Microbiology Letters* **65**, 95–100.
- VOLANAKIS, J. E. & KAPLAN, M. H. (1974). Interaction of C-reactive protein complexes with the complement system. II. Consumption of guinea pig complement by CRP complexes: requirement for human C1q. *Journal of Immunology* **113**, 9–17.
- WALKER, J. A., ALLEN, R. L., FALMAGNE, P., JOHNSON, M. K. & BOULNOIS, G. J. (1987). Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. *Infection and Immunity* **55**, 1184–1189.
- WARA, D. W. (1982). Host defense against *Streptococcus pneumoniae*: the role of the spleen. In *The Pneumococcus and the Pneumococcal Vaccine*, pp. 117–127. Edited by P. G. Quie & E. H. Kass. Chicago: Chicago University Press.
- WILMOTT, R. W., KASSAB, J. T., KILIAN, P. L., BENJAMIN, W. R., DOUGLAS, S. D. & WOOD, R. E. (1990). Increased levels of interleukin-1 in bronchoalveolar washings from children with bacterial pulmonary infections. *American Review of Respiratory Disease* **142**, 365–368.
- WINKELSTEIN, J. A. (1982). The role of complement in the host's defense against *Streptococcus pneumoniae*. In *The Pneumococcus and the Pneumococcal Vaccine*, pp. 107–116. Edited by P. G. Quie & E. H. Kass. Chicago: Chicago University Press.
- WINKELSTEIN, J. A. (1984). Complement and the host's defence against the pneumococcus. *CRC Critical Reviews in Microbiology* **11**, 187–208.
- WRIGHT, J. K., TSCHOPP, J., JATON, J. C. & ENGEL, J. (1980). Dimeric, trimeric and tetrameric complexes of immunoglobulin G fix complement. *Biochemical Journal* **187**, 775–780.
- YEAGLE, P. (1987). *The Membrane of Cells*, p. 292. London: Academic Press.
- YOTHER, J., VOLANAKIS, J. E. & BRILES, D. E. (1982). Human C-reactive protein is protective against fatal *Streptococcus pneumoniae* infection in mice. *Journal of Immunology* **128**, 2374–2376.
- YOTHER, J., MCDANIEL, L. S., CRAIN, M. J., TALKINGTON, D. F. & BRILES, D. E. (1991). Pneumococcal surface protein A: structural analysis and biological significance. In *Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci*, pp. 88–91. Edited by G. M. Dunny, P. P. Cleary & L. L. McKay. Washington, DC: American Society for Microbiology.