

Serotype-independent pneumococcal experimental vaccines that induce cellular as well as humoral immunity

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For prevention of *Streptococcus pneumoniae* (pneumococcus) infections in infancy, protein-conjugated capsular polysaccharide vaccines provide serotype-specific, antibody-mediated immunity but do not cover all of the 90+ capsule serotypes. Therefore, microbiologists have sought protective noncapsular antigens common to all strains. Alternatively, we investigated killed cells of a non-capsulated strain, which expose many such common antigens. Given to mice intranasally, this vaccine elicits antibody-independent, CD4+ T lymphocyte-dependent accelerated clearance of pneumococci of various serotypes from the nasopharynx mediated by the cytokine IL-17A. Such immunity may reproduce the natural resistance that develops in infants before capsular antibodies arise. Given by injection, the killed cell vaccine induces bifunctional immunity: plasma antibodies protective against fatal pneumonia challenge, as well as IL-17A-mediated nasopharyngeal clearance. Human testing of this inexpensive candidate vaccine by intramuscular injection is planned. Bacterial cellular vaccines are complex—a challenge for reproducibility. However, when several known protective antigens were deleted, the killed pneumococcal vaccine was still protective. This antigenic redundancy may prevent vaccine escape variants by recombinational loss, which is frequent in pneumococcus. Biochemically defined immunogens with bifunctional activity have also been devised. These immunogens are three-component conjugates in which cell wall teichoic acid (a common antigen capable of T cell activation) is coupled to a genetic fusion of two common pneumococcal proteins: a protective surface antigen and a derivative of pneumolysin, which provides TLR4 agonist activity and induces antitoxic immunity. Such constructs induce accelerated clearance when given intranasally and induce both immune mechanisms when injected. The defined composition permits analysis of structure-function activity.

B-cell immunity | T cell immunity

The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococcus) can be carried in the human nasopharynx asymptotically but opportunistically can become an agent of pneumonia and invasive infections. In industrialized nations since the antibiotics era, it is no longer—as once described by Sir William Osler—“the captain of the men of death.” However, a century after Osler's phrase, it remains a major pathogen of the elderly and immunocompromised, and it is a frequent killer of infants in poor nations. By conservative estimates, pneumococcal infections cause nearly 1 million deaths of children annually (1). Even more frequently, pneumococcus causes otitis media (infection of the middle ear cavity), which although nonfatal, is a major source of misery and hearing loss in children. Pneumococcus lacks the variety of potent toxins expressed by other pathogenic bacteria. Its cell wall and fragments, which have been characterized in great chemical detail (2–4), induce inflammatory reactions that may be responsible for much of its pathology (5–8).

History of Vaccination

The pneumococcus and immunity to its infections have been intensely studied. Beginning in the early 1900s, a number of attempts were made to use killed pneumococci as vaccines; these efforts generally suffered from lack of knowledge of antigenic structure and variation, but some efficacy against pneumonia was shown (9, 10). In the 1920–1940s, scientists at Rockefeller Institute showed that pneumococcus expresses a variety of capsular polysaccharides (CPs), that these CPs are its major virulence factor, that these CPs are antigenic (the first non-protein thus shown), that these CPs are the basis of serological type, and notably, that transformation of one type to another is specified by DNA, opening inquiry into the biochemical basis of inheritance (11, 12). The CPs impede clearance of the bacteria by phagocytic cells, and anti-CP antibodies confer serotype-specific protection by coating the capsulated bacteria to facilitate adherence by phagocytes (opsonization). Animal antisera containing CP antibody were used in therapy from about 1915 until chemotherapy became available. It was also shown that purified CPs can be used as active immunogens in adult humans; clinical efficacy against pneumonia was shown in the 1940s (13), but the approach was abandoned with the availability of antibiotics. As the limitations of antibiotics therapy were realized, vaccination with pneumococcal CP was revived in the 1960s through the efforts of Robert Austrian (14). Over 90 immunochemically distinct types are known. A 14-type mixture of the most prevalent types was selected for vaccination in 1977 and increased to 23 types in 1983 (15). This vaccine is licensed and recommended for adults >65 y and younger subjects with conditions, such as asplenia, that predispose to pneumococcal infection. Because of the extreme age dependence of the human antipolysaccharide response, however, CP vaccine is ineffective in infants (16–18). Another seminal finding of the Rockefeller group, shown with pneumococcus, was that coupling of CPs or even their immunodeterminant sugars to carrier proteins increases the CP-reactive antibody response (19, 20). Five decades later, this approach was used for conjugate CP vaccines to protect infants against the capsulated pathogen *Haemophilus influenzae* type b (21–23). Additionally, within 10 y, a vaccine against the seven most prevalent serotypes of pneumococci showed efficacy in preventing invasive pneumococcal disease in infants (24). However, the technology limits the number of serotypes that can be included, and infections caused by non-included types are a substantial problem in some populations (25). For example, in France, the overall pneumococcal meningitis incidence in children was about the same 5 y after routine use began of a seven-serotype, CP conjugate vaccine

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mixture because of serotype replacement (i.e., increased prevalence of infection by nonvaccine serotypes) (26). Conjugate mixtures with additional serotypes are being introduced but may be only of transient value; pneumococcus in its natural niche undergoes genetic change rapidly by recombination, and CP biosynthesis genes of one serotype can be replaced by genes for a differing type (27).

Noncapsular Components as Immunogens. A traditional view has been that antibodies to pneumococcal antigens other than the CP do not confer immunity effectively because of masking by the capsule; thus, the differing serotypes are, in effect, independent pathogens regarding immunity (28). More recent work, however, has challenged this view. Nasopharyngeal colonization is a prerequisite of pneumococcal infection (29). Colonization is a complex process in which differing host cell receptors are engaged by pneumococcal components other than the CP (30, 31). When isolated from the bloodstream, pneumococci are highly capsulated; when isolated from the nasopharynx, subcapsular structures, such as the cell wall teichoic acid is more exposed and indeed, needs to be surface-expressed for interaction with receptors on mucosal cells (32, 33). Beginning with the works of Briles and Tomasz (34), Briles et al. (35), and Paton et al. (36), pneumococcal antigens with immunoprotective potential have been identified that are common to many or all serotypes. With the advent of genomic methods, many of these common antigens have been described and evaluated (review in ref. 37). When tested singly, antibodies to such antigens can be protective in animal models; these antibodies seem less potent than capsular antibody, but they can be additive or even synergistic when more than one specificity is used together (38, 39). There have been clinical trials of some of these antibodies as purified proteins (40–42), and attenuated *Salmonella typhi* engineered to express one such protein (43, 44) is currently in phase I testing as an oral vaccine. However, none have yet shown protection in humans, much less in infancy. There is a hypothetical disadvantage of immunity evasion if a single common protein is used. As mentioned above, pneumococcus is highly diverse genetically (45, 46) and capable of rapid recombinational change. A protein that is not essential for viability but significant in immunological recognition by the host can be altered or eliminated (e.g., a major recent study addressing the issue described two strains in which surface proteins with vaccine potential were not expressed) (27). Candidate vaccines combining several common proteins (42, 47), in addition to gaining potency, may reduce the possibility that pneumococcus would readily evolve to evade immunity induced by vaccination.

Reconsideration of a Whole-Cell Immunogen. Seeking an economical means of presenting a multiplicity of common antigens in native configuration without masking by the CP, we and colleagues have investigated immunization with killed, non-capsulated pneumococcal cells (WCA). The original approach was to vaccinate intranasally (i.n.), intending to reduce colonization by raising mucosal antibodies to many of the common antigens. In general, bacteria-like particles have an advantage in mucosal immunization (48, 49); one explanation is that particles copresenting antigens and agonists of toll-like receptors (TLR; pattern recognition receptors of the innate immune system) are processed for immunity, whereas soluble antigens tend to be processed for tolerance (50). In our initial studies, WCA given to mice i.n. with mucosal adjuvants accelerated the clearance of capsulated pneumococci of the several serotypes tested (51, 52).

The antigenic specificity of the protection is not defined. Electrophoresis with Western blotting (to separate and show components reactive with WCA antiserum) shows that antibodies to several known protective proteins plus dozens of not yet identified proteins are elicited as expected. There is considerable

redundancy (e.g., the teichoic acids or the entire class of choline-binding proteins can be removed from the WCA and it still elicits protection). This redundancy lessens the probability that alteration or deletion of proteins by recombination would permit pneumococcus to evade the WCA-induced immunity.

Unexpected Mechanism Revealed by Mucosal Vaccination. Although plasma antibody responses were induced in normal mice, the clearance unexpectedly was inducible in $\mu\text{MT}^{-/-}$ mice (53), which congenitally lack antibody responses. Furthermore, this immunization failed in nude mice (lacking T lymphocytes) and MHC II-deficient mice (lacking CD4+ T cell responses), but it worked normally in MHC I-deficient mice (lacking CD8+ T cell responses) (53). The antibody independence and T cell dependence were surprising, because pneumococci are considered extracellular pathogens (killed when phagocytosed) in contrast to microbes such as mycobacteria that can persist within phagocytes and require activated T cells for clearance (28). Thus, the mediator of adaptive immunity to pneumococci had been considered to be opsonic antibody. Our surprising observations on the immunity induced by i.n. vaccination with WCA were pre-saged by studies of pneumococci in naive mice: the cellular response to pneumococcal infection had suggested a role of CD4+ T cells in the control of pneumonia and blood-borne infections (54), and the gradual clearance after intranasal challenge was similar in $\mu\text{MT}^{-/-}$ and normal animals (55). The observation that (human) children naturally increase their immunity to pneumococci of many serotypes simultaneously and before detectable capsular antibodies arise prompted our suggestion that an antibody-independent, CD4+ T cell-dependent process is an early contributor to natural pneumococcal immunity (53, 56).

The cytokine IL-17A, a product of the Th17 cell lineage, was known to activate neutrophils and be an effector in immunity to pathogens such as fungi, *Klebsiella pneumoniae*, *Bordetella pertussis*, and *Mycobacterium tuberculosis* (57–61). We showed that IL-17A likewise is an effector of the WCA-induced acceleration of pneumococcal clearance in mice. (i) The T cells of vaccinated mice become primed for production of IL-17A in response to pneumococcal antigens in cell culture. The immunity to colonization induced in individual mice correlated well with the IL-17A concentration that can be elicited by WCA in samples of their blood taken just before colonization challenge. (ii) Mice lacking the receptor for IL-17A (and neutrophil-depleted mice) were not protected (62). Thus, WCA given by the i.n. route to mice induces an antibody-independent, IL-17A-mediated immunity to colonization. It was also found that human IL-17A stimulates killing of pneumococci in vitro by human neutrophils in the absence of antibodies and complement; additionally, production of IL-17A in culture by T cells from umbilical cord blood from human neonates was extremely low but was substantially higher in cell cultures from older subjects (62). A protective role of IL-17A was subsequently also shown in the clearance of pneumococcal colonization in the absence of immunization (63). Details of the Th17 protective cytokine pathway have been recently reviewed (64).

These observations support the idea that, before development of antibody, early life natural immunity to pneumococci includes increased priming for IL-17A production on contact with the bacterium. Vaccination of infants with WCA may hasten this development and thus, the ability to clear pneumococci from the nasopharynx.

Parenteral Vaccination. The immunization of mice to accelerate clearance can be performed i.n. or by other mucosal routes such as buccal or sublingual; any of these routes requires a mucosal adjuvant for which enterotoxins such as cholera or nontoxic mutated derivatives have been used in our murine studies (65). Although there are potential difficulties with such adjuvants by

the intranasal route in humans because of the possibility of facial nerve paralysis (66), the ease and economy of mucosal administration might encourage future vaccination programs to consider this approach. For childhood vaccines in developing nation programs, however, the World Health Organization's "Target Product Profile" presently prefers vaccines to be given by injection. Therefore, WCA was also studied in mice by injection with adsorption to aluminum adjuvants, which are routinely and safely used in human vaccination (67). These mice similarly displayed accelerated nasopharyngeal clearance, which correlated with their priming for IL-17A responses. Moreover, their plasma antibody responses were >30-fold higher than if nasally immunized, and they were also protected in our model of lethal aspiration pneumonia from type 3 or 5 pneumococci (67). In the lethal model, protection was related to IgG antibody response and was not abrogated by depletion of CD4+ T cells at the time of challenge (67). WCA antiserum raised by injection in rabbits was passively protective in the aspiration pneumonia model (i.e., immunity could be transferred to naive mice with serum). Thus, parenteral immunization of mice with WCA revealed a bifunctional immunity: with a nonlethal intranasal challenge, clearance is CD4-dependent and IL-17A-mediated; in a lethal aspiration pneumonia model, survival is CD4-independent and dependent on plasma antibodies (67). Injection of WCA, therefore, might reduce the probability of invasion by accelerating clearance and may also protect systemically in case the mucosal barrier were breached. Through the substantial support and input from the health organization PATH, WCA has been made under good manufacturing practice at Instituto Butantan and Walter Reed Army Institute for Medical Research, and authorization for human trials by parenteral injection has been sought from the US Food and Drug Administration.

Strains Coverage. As mentioned, pneumococci are genetically and antigenically highly variable, and one must consider whether a vaccine made from a single strain would cover the extant variety of pneumococci worldwide and variants that might arise with the pressure of WCA-induced immunity. This variety cannot be dealt with by animal protection studies, because relatively few pneumococcal serotypes reliably infect animals in models that recapitulate human disease. We have thus far addressed the issue with two immunologic assays. (i) The antibody reactivity was tested with the rabbit WCA antiserum, comparing it with preimmunization serum in a capture ELISA conducted with a selection of 24 strains, including 15 serotypes and 12 multilocus sequence types (of which 14 were isolates from invasive diseases and 10 were from carriage). WCA induced elevated titers against all of the strains (fold rise ranged between 10 and 140 times higher than the control serum). (ii) Priming for Th17 response was tested by immunizing mice with WCA, isolating their splenocytes, and measuring IL-17A production by these cells in culture when stimulated by the various strains (68). Stimulation was consistent, indicating that the capsulation of the strains did not interfere with expression of antigens able to stimulate T cells primed with the acapsular vaccine cells. It, thus, is reasonable to expect broad coverage by WCA.

Fractionation. The WCA can be prepared so as to retain proteins released from the killed cells. The soluble fraction contributes to immunity differentially from the cellular fraction: when separated and adsorbed to Al(OH)₃, the soluble fraction exceeds the cellular fraction in antibody induction but is somewhat less active in IL-17A induction. In recent efforts using tools from

proteomics and chromatography, the WCA soluble fraction was used as a source for the identification of pneumococcal antigens that, when administered alone, could confer protection against pneumococcal colonization in mice. Several such proteins have been identified (47). These findings are presently the limit of structure-function information on the WCA. We hope to develop additional avenues of analysis.

A Semisynthetic Antigen Inducing Bifunctional Immunity. From the viewpoint of vaccine manufacturers, whole bacterial vaccines are disadvantageous because of antigenic complexity and the challenge of reproducibility. Therefore, we and colleagues are attempting also to devise biochemically defined pneumococcal constructs with a similar bifunctional potential. The cell wall polysaccharide (CWPS) of pneumococcus (structurally, a teichoic acid) is a highly conserved, serotype-independent antigen with a zwitterionic structure that confers the capacity for T cell activation and IL-17A elicitation (69–72). Administered i.n. to mice as a stand-alone antigen, CWPS induces IL-17A-mediated clearance of pneumococci similarly to WCA (72, 73). Noting this feature, the work by Lu et al. (73, 75) coupled CWPS to a fusion protein, combining portions of the protein pneumococcal surface adhesin A (known to induce resistance to colonization) and a genetically detoxified variant of the cholesterol-dependent toxin pneumolysin (known to induce protection against systemic toxic effects of pneumococcus as well as be a TLR4 agonist) (74). The immunogenicity of this three-component construct [fusion conjugate (FC)] exceeded the immunogenicity of a simple mixture or any of the respective three two-component constructs (75). Like WCA, FC, when injected, was protective in both the colonization and lethal pneumonia models. Structure-immunogenicity relationships can be examined more readily in FC than WCA. For example, substitution of dextran for CWPS as the polysaccharide component diminishes the protection against sepsis but not colonization. Additional studies to define the structural basis of the bifunctional immunity are planned (e.g., surface proteins other than pneumococcal surface adhesin A can be evaluated).

Prospects. In the past decade, increasing attention of health agencies and philanthropic organizations has become focused on prevention of pneumococcal disease in developing nations. Immunologically, these infections are preventable, but the optimal strategy for so doing includes programmatic as well as immunological challenges. The efficacy of CP conjugate vaccines against invasive disease due to of included serotypes is well-established. A number of potentially more inexpensive, serotype-independent candidate vaccines (including our vaccine) are being energetically pursued, but studies to establish their equivalence or superiority to the CP conjugates will raise issues yet to be resolved. The initiating motivation of vaccine cost is becoming a less crucial issue, because it is realized to be a small portion of the total expense of a vaccination program, and philanthropic organizations' purchase of vaccines is increasing. More important may be the possible transience of protection in a population by CP conjugates caused by serotype replacement, a complex issue to analyze. The late Robert Austrian is rightly credited with reviving CP vaccination to address the limitations of chemotherapy; however, his more enduring legacy may be an insistence on looking hard at the numbers in dealing with the pneumococcus (14). There is considerable scientific and financial commitment to address the problem, and it will be interesting to see how the solution evolves.

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