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MYCOBACTERIAL PATHOGENESIS: A HISTORICAL PERSPECTIVE

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1. ABSTRACT

Tuberculosis is an age-old human affliction which continues to flourish worldwide despite the development of effective drugs for its treatment and a vaccine (BCG) for its prevention. At least 8 million people die from this disease each year, a figure which is likely to increase as the AIDS epidemic continues its relentless spread into Africa and Southeast Asia. Consumption was shown to be caused by *Mycobacterium tuberculosis* more than a century ago, yet we still know very little about the mechanisms used by this organism to elude the normally effective cellular host defenses as it establishes a progressive infection within the lung. The majority of individuals exposed to tuberculous infection are able to limit the primary infection to the lungs and its lymph nodes, resulting in a latent form of the disease which can provide the host with a lifelong immunity to reinfection. While a great deal is known about the cellular mediators of this immune response (together with the cytokines which modulate them) we lack a clear understanding of the role that they play during the establishment of the dormant form of the disease.

Live BCG vaccine has been widely used in many Third World countries as a major component of their tuberculosis control programs. However, several carefully controlled human trials have shown little protection achieved in vaccinated individuals. Development of improved vaccines, both for the prevention and therapy of this disease is an urgent research priority and a number of potential immunogens are under active investigation. However, our limited understanding of the pathogenesis of this chronic disease, together with a lack of data on the role played by different bacterial components in the modulation of the immune response, continues to severely limit our ability to develop a rational approach to this project. To achieve this goal, it will be necessary to establish innovative approaches to the presentation of protective antigens by taking advantage of recent advances in the molecular biology of this complex and enigmatic group of organisms.

2. INTRODUCTION

Pulmonary tuberculosis has been recognized as an important, life-threatening human disease since the beginning of recorded history (1). Despite substantial advances made over the past 100 years in the treatment and prevention of this disease, it continues to kill nearly three million of people each year, making it the most important bacterial infection we face in the world today (2). According to the World Health Organization, as many as a third of the world's population (1.7 billion people) are presently infected with tubercle bacilli, resulting in nearly 8 million new cases of this disease being reported each year. Based on present trends, this yearly figure is expected to rise to more than 10 million by the year 2000 (3). Even in the United States, where tuberculosis was once thought to be on the brink of elimination (4), it is estimated that 10 to 15 million Americans are still infected with tubercle bacilli and therefore at risk of developing reactivational disease (5). After a steady decline over several decades, the number of new tuberculosis cases reported to the Centers for Disease Control in Atlanta, underwent a sharp increase beginning in 1985, a shift which represents an excess of more than 52,000 new cases over that predicted on the basis of the earlier rate of decline. Much of this increase has been a direct result of the worldwide AIDS epidemic, which reactivates latent cases of tuberculosis with disastrous results for the doubly infected individual (6). The combined *M. tuberculosi*s and HIV infections constitute a particularly deadly combination with sharply increased mortality rates for both diseases. While the number of new cases of tuberculosis for the United States as a whole is once again on the decline (largely as a result of strenuous public health control measures), the numbers continue to rise in several States, as well as the District of Columbia, where the disease remains at near epidemic levels (7, 8). Some communities report that a quarter to a third of their tuberculosis patients are coinfected with HIV (9) and since many AIDS patients live in homeless shelters, prisons or hospice settings, they are also at increased risk of acquiring multidrug-resistant tuberculosis infections (10). As long as the AIDS epidemic continues to spread (11),

tuberculosis will remain a serious public health problem in this country, as well as overseas (12).

Pulmonary tuberculosis is a chronic, debilitating lung disease, mainly caused by *M. tuberculosis* (13). Most cases of tuberculosis are the result of direct person-to-person spread by the *M. tuberculosis* as they are coughed out by an individual suffering from open cavitary lung disease (14). The tubercle bacilli are expelled into the air in tiny droplets 3 - 5 microns in diameter which can travel surprising distances in still air, infecting other people living or working with the index case (15). Some strains are unbelievably infectious (16), especially in a closed environment such as occurs in prison or in a nursing home (17). For obvious reasons, it has been difficult to establish the minimum size of an infectious dose for human patients, but guinea pig studies suggest that active disease can occur following the inhalation of only 1 or 2 viable tubercle bacilli (15, 18). On entering the alveolus, the organisms are taken up by the resident alveolar macrophages where they begin to multiply (19). Some of these organisms will be carried to the hilar and tracheobronchial lymph nodes where they will establish secondary tubercles. Most immunocompetent adults (90-95%) are able to confine the infection to these draining lymph node(s) allowing the latent form of this disease to develop. In less fortunate individuals, the infection spreads via the bloodstream to the spleen, the kidneys, the bone marrow and the central nervous system (frequently seen in infants and children whose cellular defenses have not yet become fully developed). The developing infection may continue to spread despite the activation of a strong cell-mediated immune response which nevertheless, seems incapable of limiting the developing infection within the lung (20). Regardless of the effectiveness of the immune response, it may not eliminate all the viable organisms from the tissues (21). Thus, in many patients, a small number of viable tubercle bacilli may survive *in vivo* for years or even decades after the primary infection has apparently been brought under control. Despite the obvious immunological importance of this phenomenon (22), we still know very little about the mechanism(s) involved or even the form that the organisms take whilst in this dormant state (21). The latent infection may provide the host with an increased resistance to second attacks of this disease (23), but at the same time, it can also constitute an important source of reactivational disease late in life, or following an HIV infection (17, 24).

3. CONSUMPTION, PHTHISIS, SCROFULOSIS, POTT'S DISEASE OR THE WHITE PLAGUE

Pulmonary tuberculosis was first recognized as a debilitating lung disease during the classical Greek era (1). References to Consumption can also be found in a number of ancient Hindu texts. However, the infectious nature of this disease was not recognized until Villemin successfully infected rabbits with blood and sputum taken from his tuberculous patients in 1868. The implications arising from his findings ran contrary to the conventional medical wisdom of the time and were almost uniformly ignored by his colleagues who continued to believe that consumption was caused by breathing unhealthy air (miasmas), by bad living habits, even a result of moral turpitude. This attitude only changed after Robert Koch reported in 1882 that he had successfully cultured the tubercle bacillus on potato and inspissated serum slants in his laboratory (25). Koch also injected his pure culture into rabbits and guinea pigs which developed active tuberculosis, thereby satisfying his famous set of postulates as the causative organism of this disease. By injecting cultures obtained from patients exhibiting the many clinical manifestations of this disease, he proved beyond all doubt that *M. tuberculosis* was the sole cause of this highly infectious human disease. Despite this masterful exercise in experimental pathology, Virchow who was the most influential pathologist in Berlin at that time, steadfastly refused to accept the validity of Koch's findings. His resistance was also responsible for the publication of this milestone paper in a relatively obscure medical journal which was not readily available to the majority of physicians and academicians in the United States.

Abstracts of this paper appeared in a number of British and American medical journals where they attracted the attention of a young New York physician, Edward Livingston Trudeau, who was suffering from advanced pulmonary tuberculosis (26). At that time, Dr. Trudeau was living in a remote village in the Adirondack Mountains in Northern New York State for the sake of his health. He had contracted tuberculosis in 1865 while nursing his elder brother who died from miliary tuberculosis. While tending his patient, Trudeau had no idea that the disease was contagious and had taken no precautions against his own infection. As a result, several years later he developed a persistent fever and cough and on consulting a colleague, was informed that he had massive upper lobe tuberculous involvement. Trudeau was well aware from his own medical training what this meant and he commented in his autobiography that his feelings were akin to the prisoner at the bar who is told he is about to be hanged (26). Within a year he was too sick to continue his medical practice and decided to spend his few remaining months of life hunting and fishing at Paul Smith's Camp on Lake St. Regis in the Adirondacks. At first, he was too weak to walk more than a few steps and had to be carried on a litter by his guides to a suitable hunting spot. However, by the end of the summer, Trudeau's health had improved substantially as a result of a regimen of rest, good food and clean mountain air. His near miraculous recovery led Trudeau to move his family to Saranac Lake, where his health continued to improve to the point that he was able to resume the practice of medicine.

About this time he was given an English translation of Koch's paper which Trudeau later described as one of the most important medical papers ever written (26). In particular, he was struck by the incisive experimental approach used by Koch and resolved to isolate his own culture of tubercle bacilli so he could carry out his own experimental studies into the course of this enigmatic disease. His first step was to learn how to stain and culture this demanding pathogen in the laboratory, using his own clinical material for the purpose. Then, he established a diagnostic laboratory in his house and set about studying the role of various environmental factors in the establishment of a tuberculous infection in rabbits, using the pure culture of *M. tuberculosis* which he had recently isolated from his own

sputum (27). At that time, a bitter controversy was raging over the role played by environmental factors in the establishment of tuberculosis in people. Trudeau set up his first experiment in which he infected groups of rabbits with tubercle bacilli and then housed them under different living conditions. He placed his first group of uninfected controls in a damp, dark pit with only minimal food and water. A second group of animals were infected with tubercle bacilli and kept in a dark, crowded box in an unheated cellar. The third group were also infected and released on a small island in Lake St. Regis where they had plenty of food and exposure to fresh air and sunlight. When Trudeau examined his three groups of rabbits several months later, the results were clear cut and the conclusions indisputable. The infected rabbits which he kept in the cellar were all dead as a result of progressive tuberculous disease whereas the uninfected rabbits living under the same conditions were emaciated but otherwise alive and well. Of the infected rabbits which he had released on Rabbit Island, one died in the first month but the other 4 were alive and well with no sign of tuberculous disease when they were sacrificed 6 months later (27). The results of this simple experiment vindicated Trudeau's belief that the progress of a tuberculous infection could be arrested, even reversed, by a regimen of rest, good food, sunlight and fresh air. He had also established that poor housing and malnutrition were not, *per se*, a cause of tuberculosis.

In retrospect, this simple experiment represented a remarkable *tour-de-force* for a young country doctor with little or no formal laboratory training and who, on his own initiative, established an active research program at the Adirondack Cottage Sanatorium only one year after it first opened its doors in 1884. Throughout his life, Trudeau remained convinced that effective treatment of this disease depended on a combination of clinical and experimental research, a philosophy which undoubtedly helped to establish the Trudeau Laboratory as a leading tuberculosis research center in the United States.

One of the most enduring dreams in tuberculosis research has been the development of an effective, fully protective vaccine against this highly infectious pathogen. At the time that Trudeau began his studies in tuberculous rabbits, the mortality rate for this disease in the United States was around 500 cases per 100,000 persons per year, with survival rates ranging from 1 in 3 to 1 in 4 for most of these unfortunate patients. Survival of infected infants and young children was even more dismal. In the absence of any known cure, the best alternative seemed to be preventive by the use of vaccination procedures along the lines successfully developed by Pasteur against rabies and fowl cholera. During his early studies, Koch (28) tested a number of inactivated products prepared from the tubercle bacillus and announced in 1891 that injections of an extract which he called old Tuberculin" showed therapeutic promise when injected into tuberculous patients. The rationale behind this treatment was based on his demonstration that the injection of killed tubercle bacilli into the skin of tuberculous guinea pigs resulted in an accelerated inflammatory response with local necrosis (the so-called Koch reaction). Koch reasoned that a similar reaction would occur within the lungs of tuberculous patients receiving intravenous injections of Old Tuberculin and he assumed that this would have some therapeutic benefit for the patient. Trudeau and his colleagues were quick to repeat Koch's study but after seeing a number of fatal complications (due to tuberculin shock), they concluded that such therapeutic injections were too dangerous and discontinued the treatment (29). Despite this early disappointment, Old Tuberculin remained a useful diagnostic reagent for more than half a century before its cellular nature was first established by Chase and Landsteiner (30).

During his early tuberculin therapy studies, Trudeau had tried vaccinating guinea pigs and rabbits with killed suspensions of tubercle bacilli and their extracts, but without much success. Like many pathologists, Trudeau had been struck by the presence of old, healed tuberculous lesions in the lymph nodes of many apparently healthy adults who had died from non-tuberculous causes (31). Trudeau assumed that these individuals had been infected with tubercle bacilli early in life and had developed the latent form of the disease in which they continued to express a positive tuberculin skin reactivity but with no signs of active, progressive disease. Since virtually everyone at that time was being exposed to individuals with active pulmonary disease, Trudeau reasoned that latent tuberculosis somehow protected the individual against secondary attacks of the disease. If dead bacilli were unable to protect vaccinated animals from a tuberculous challenge, Trudeau reasoned that the living organism must possess protective factors not present in killed bacilli. This conclusion seemed consistent with his earlier finding that live *M. avium* provided excellent cross-protection to rabbits challenged with virulent *M. tuberculosis* (32). However, *M. avium* was too virulent to use in humans and so Trudeau turned his attention to an attenuated strain of *M. tuberculosis* R1. At that time, there was no method to preserve *M. tuberculosis* cultures in the laboratory. Clinical isolates were transferred onto fresh media every 3 or 4 weeks, a procedure which usually resulted in a slow loss of virulence for experimental animals (and presumably, for people). The usual procedure was to discard such cultures and replace them with a fresh clinical isolate, of which there was no shortage. However, a few of these old strains had been retained in the Trudeau Laboratory collection, usually as a source of Old Tuberculin. *M. tuberculosis* strain R1 had been maintained in potato-glycerine-peptone broth for 11 years, by which time it was no longer able to kill rabbits or guinea pigs even when injected in massive doses. Trudeau inoculated a number of rabbits and guinea pigs with two doses of the live R1 vaccine and challenged them 3 months later with a highly virulent strain of *M. tuberculosis* (32). All of the vaccinated animals were disease-free when examined 12 months later, whereas the unvaccinated controls were all dead within 60 days of challenge. Such a level of protection was highly impressive, yet there is no indication that Trudeau gave serious consideration to testing his R1 vaccine in humans. Years later, Steenken re-examined several of these *M. tuberculosis* strains for their immunogenicity in guinea pigs and concluded that they could be potentially useful as vaccines for silicotic miners who were known to be extraordinarily susceptible to tuberculous infection (33). However, there is no recorded attempt to translate these experimental findings into clinical testing in human volunteers and it remained for Calmette and Guerin to achieve this goal with their attenuated BCG strain of *M. bovis* (34).

4. BACILLE DE CALMETTE-GUERIN (BCG) VACCINE DEVELOPMENT

BCG vaccine was determined to be safe and effective when administered orally to infants and young children with few serious side effects (35). However, this conclusion was almost completely negated by the Lubeck disaster in which 72 infants died after being fed vaccine which had been accidentally contaminated with virulent tubercle bacilli. This reputation was not helped by the dismal failure of several well-controlled BCG field trials carried out in areas known to suffer from a high level of endemic disease (36). A recent meta-analysis of this published data has concluded that the level of protection achieved as a result of BCG vaccination averaged around 50%, with substantially lower levels being achieved in countries lying near the equator, where, ironically, the need for effective protection against this disease is the greatest (37). The reasons for the observed lack of anti-tuberculous protection in these trials remains controversial, since the same vaccines showed high levels of protective activity when tested in Britain (38).

A number of experimental studies had indicated that specific antibodies played little role in the expression of acquired anti-tuberculous immunity (39, 40). However, the nature of the protective mechanism remained a mystery until the pivotal studies of Max Lurie in tuberculous rabbits (41). Lurie infected his rabbits with virulent strains of *M. tuberculosis* or *M. bovis* and followed the progress of the resulting pulmonary disease. During these studies, he determined that the rabbit infection showed a number of similarities to the naturally acquired human disease. Then, in a series of landmark studies, Lurie examined the course of this disease as it developed in rabbits bred for their innate susceptibility or resistance to tuberculosis (42). In the course of these studies, Lurie was able to establish for the first time that anti-tuberculous immunity was mediated by a population of immunologically activated macrophages (43). This was achieved by following the growth of tubercle bacilli introduced into the anterior chamber of the rabbit's eye, together with activated macrophages harvested from naive or BCG vaccinated donors. Control animals receiving monocytes from animals immunized with killed tubercle bacilli were unable to kill virulent tubercle bacilli, thereby demonstrating the crucial role played by the living organism in the induction of an effective immunity to a tuberculous challenge (41). These studies were all the more remarkable because they were carried out before *in vitro* tissue culture techniques became widely available and more than a decade passed before Lurie's findings could be confirmed using this newly developed technique (44). These *in vitro* studies ushered in the era of cell-mediated immunity as a major arm of the host defense against a variety of intracellular pathogens (45).

The pathogenesis of pulmonary tuberculosis as it develops in the rabbit model was extensively described by Dannenberg, based on his extensive histological examinations of the resulting lesions (46). In many of these studies, the animals were exposed to a small aerogenic challenge with virulent bovine tubercle bacilli and the progressive development of the resulting disease was divided into 4 main phases:

a). Phagocytic uptake of the organisms by the resident alveolar macrophages which carry them to the hilar and mediastinal lymph nodes.

b). Logarithmic growth by the organisms occurs at both the original infection site and in the draining lymph nodes for a 3 to 4 week period.

c). Tuberculin skin hypersensitivity and cell-mediated immunity, mediated by separate populations of specifically sensitized T-cells, develop in the infected host and the resulting activated monocytes slow or even reverse the growth of the pathogen within the tissues.

d). The developing tubercle may undergo progressive caseation, often followed by internal liquefaction due to the hydrolytic enzymes released by the activated macrophages. The resulting fluid provides a highly nutritious growth medium for the tubercle bacilli which begin to increase in number. Eventually, the cavity will enlarge until it erodes into a bronchiole and the entry of air results in an explosive growth of the tubercle bacilli. Many of the organisms are carried up with the sputum and expelled into the air as infectious droplets during coughing or spitting (14).

A bitter controversy has developed over the last 50 years regarding the relationship between tuberculin hypersensitivity and acquired cell-mediated immunity in the tuberculous host (47). Much of this debate stems from the apparent dissociation of the two phenomena in vaccinated animals subjected to a number of experimental procedures. Mackaness (48) pointed out the fallacy in many of these arguments, showing that tuberculin skin hypersensitivity is a poor and unreliable indicator of the presence of a protective cell-mediated immunity within the lungs and spleen of the immunized host. With the availability of inbred mouse strains differing in their innate resistance to tuberculous challenge (49), it became possible to adoptively immunize mice with purified T-cell suspensions harvested from immunized donors (50). These studies confirmed that these two cellular responses are mediated by different T-cell subsets which can now be readily distinguished and quantitated on the basis of their specific cell surface markers (51, 52). The explosion of new data stemming from studies of this nature has dramatically increased our understanding of the cellular and molecular mediators of this anti-tuberculous immune response (53). The skin test anergy characteristic of many patients suffering from advanced systemic disease may simply reflect the inability of a minute amount of PPD injected into the skin to be recognized by the circulating sensitized T-cells in the presence of the great excess of mycobacterial antigens already present in the heavily infected host. The ability of splenic T-cells harvested from anergic donors to transfer tuberculin hypersensitivity as well as adoptively immunize naive recipients against infection with virulent organisms would seem to confirm this conclusion (51).

Development of delayed hypersensitivity by the infected host seems to be temporally related to the evolution of a protective cell-mediated immune response, suggesting that the two processes may be functionally linked in the naturally infected host (54). However, this conclusion is not consistent with recent studies carried out in ICAM-1 knock-out mice (55). On the other hand, a prolonged state of

tuberculin hypersensitivity seems to be associated with severe and progressive tissue damage of the kind usually associated with patients suffering from advanced clinical disease (19, 56). It seems that a little tuberculin hypersensitivity may be good for you by drawing large numbers of blood monocytes into the developing tubercle. However, prolonged hypersensitivity leads to the destruction of functional lung tissue, presumably as a result of excessive IFN-γ and TNF–α production (57). For optimal protection, a delicate balance must be struck between competing cytokine populations as they are released within the developing tubercle (53). This balance may vary depending on the organ (or even tissue), the virulence of the pathogen and the stage of the infection (58). Other factors involved in this process are likely to be the age and nutritional status of the host, the presence of physical or mental stress, and the existence of an intercurrent infection (24, 59). The recent advances made in our understanding of cytokine biology have moved the immune response from a cellular to a molecular level, but we still know surprisingly little about the host-parasite interactions which occur during the crucially important, initial stages of the infection (60). Much of this ignorance stems from an inability to decide just which antigens (or epitopes) are responsible for the induction of the fully protective immune response, or even which antigens are responsible for the maintenance of a long-lived memory immune response (61). The dominant T-cell population is known to undergo a series of changes as the infection first develops and later declines under the influence of the emerging immune response. However, we still do not know whether this immune memory depends on the presence of a residual bacterial infection within the lymphoreticular organs of the convalescent host or whether these cells can persist in the absence of a latent infection (62). The kinetics of this memory T-cell response is likely to be the subject of intensive immunological investigation for some time to come.

5. VIRULENCE FACTORS ASSOCIATED WITH THE TUBERCLE BACILLUS

M. tuberculosis possesses an array of unique and relatively toxic sulfolipids, glycolipids and lipo-oligosaccharides, many of which may serve as virulence factors in experimental animals (63, 64). Some of these factors may only be produced in significant amounts while the organism is growing *in vivo*, since it is known that bacteria isolated from host tissues can differ in a number of ways from their *in vitro*-grown counterparts (65). However, despite a great deal of study, we still know surprisingly little about their role as virulence factors (genes, antigens, toxins, enzymes) or how they are able to assist the growth of the pathogen within an intracellular environment. Comparison of the genetic makeup of *M. tuberculosis* strains H37Rv and H37Ra, or *M. bovis* strains Ravenel and BCG, indicate a substantial loss of genetic material by the attenuated organisms, although the significance of the lost genes has yet to be established in terms of pathogenicity (66, 67).

In contrast to the elegance and sophistication of the genetic approaches used in these studies (68), the resulting vaccine must be tested for its residual virulence and immunogenicity by methods which have changed little in over half a century (69). Traditionally, virulence tests are carried out in guinea pigs which have long been known to be exquisitely susceptible to parenteral challenge with tubercle bacilli. Many of the early protection studies merely compared the mean time to death in vaccinated vs. control animals following a large intratesticular (70) or intravenous (71) challenge dose of *M. tuberculosis* H37Rv. This methodology was refined by recording the amount of tuberculous involvement (number of surface tubercles) seen in the spleen, liver and lung using an arbitrary semi-qualitative scale (70, 72). Later, Mitchison transformed this organ involvement into a root mean index of virulence (ranging from 0.0 for *M. tuberculosis* H37Ra to 1.2 for Erdman) as a more quantitative assessment of the level of organ involvement (73). Finally, Middlebrook (74) and Smith (75) introduced the use an aerogenic challenge inoculum to increase the relevance of the infection to the naturally acquired human disease. Using a Middlebrook chamber, guinea pigs can be reliably infected with as few as 2 or 3 viable *M. tuberculosis* Erdman, with most of the control animals dying within 100 days from a fulminant systemic infection (76). On the other hand, the BCG vaccinated animals were able to survive for at least 400 days. Smith also used X-ray examination of the lungs and the quantitation of the bacterial population within the lung and spleen to further increase the sensitivity of the methodology (77). There is little question that these refinements greatly increased the sensitivity of the assay. However, these results may be somewhat misleading because the guinea pig is too susceptible to a tuberculous challenge to serve as an accurate model of the human disease, in which the majority of adults are relatively resistant to this infection (78).

Mice have been used in tuberculosis research ever since Koch first demonstrated that they were susceptible to experimental infection by virulent tubercle bacilli (49). Rodents are not naturally subject to tuberculous disease, with the possible exception of *M. microti*, which was first isolated from the field vole (79). Early mouse studies used large challenge inocula of *M. tuberculosis* which killed the controls after 3 or 4 weeks (71). In this model, protection was measured as the increase in survival seen in the vaccinated compared to the control mice after 30 days. With the development of 7H10 agar culture medium (80), it became possible to follow the growth of a sublethal challenge inoculum in the lung and spleen at intervals throughout the infection (81, 82). Counts carried out in BCG vaccinated mice required the use of thiophene carboxylic acid hydrazide, or acriflavine to the agar to inhibit the growth of any residual BCG (83, 84). In a classic series of studies, Dubos and his colleagues compared the immunogenicity of a number of BCG substrains by quantitating the growth of both the vaccine and challenge populations in selected host organs throughout the entire experimental period (82). While this type of experimental protocol is extremely time consuming and tedious, it demonstrates the presence of a protective antibacterial immune response within the vaccinated host (85). With the availability of inbred mouse strains showing different levels of innate susceptibility to tuberculosis as well as the reagents needed to identify and sort splenic T-cell subsets, it became possible to adoptively protect naive recipients against an aerogenic tuberculous challenge with

pure suspensions of specifically sensitized T lymphocytes (86). These experiments have conclusively demonstrated the pivotal role played by these T-cells in the expression of acquired anti-tuberculous immunity (87). These studies have led to an explosion of new immunological information, allowing a more sophisticated approach to the development of protective reagents against a variety of intracellular pathogens (88). Attempts to further improve vaccines for use in the prevention and immunotherapy of this tenacious disease will constitute a major aspect of future studies with this important human pathogen (89).

6. ANTI-TUBERCULOSIS VACCINES AND PROTECTION AGAINST AEROGENIC INFECTION

The incidence of tuberculosis in the United States has changed dramatically over the past century from a disease affecting mainly infants and young adults in large urban communities, to one occurring mainly in health care settings such as nursing homes and AIDS hospices, in homeless shelters and in prisons (17, 90). While case-finding and chemotherapy has resulted in a spectacular drop in mortality rates for this disease in Europe and North America, it has had less effect in Third World countries where tuberculosis has become an increasingly serious public health problem (3). This shift probably reflects the crushing poverty, overcrowding, nutritional deficiencies and lack of financial and medical resources associated with these countries (91). The severity of the problem has undoubtedly been exacerbated by the HIV epidemic which continues to sweep through Africa, Asia and much of Latin America (11). The increasing incidence of multidrug-resistance seen in *M. tuberculosis* isolates recovered from many of these patients is even more worrying (92). Contrary to earlier conventional wisdom, these multiply resistant mutants have not lost their virulence for AIDS patients, many of whom develop an acute, virtually untreatable, life-threatening disease. Under these circumstances, tuberculosis is likely to remain a serious world problem for a long time to come and in the absence of more effective anti-tuberculous drugs, the only alternative strategy would seem to be the development of more effective vaccines, both for the prevention and immunotherapy of this tenacious disease (89, 93).

Live BCG vaccine has been widely used over the past 50 years to immunize infants and young children (mostly in Third World countries) with few serious side effects (36). However, the efficacy of this vaccination has been questioned following the failure of several carefully controlled human vaccine trials (94, 95). Thus, the decision by many governments to continue using a potentially flawed vaccine has been based primarily on its low cost, ease of production, storage and administration. However, a recent meta-analysis of the protection achieved in a number of BCG trials concluded that neonatal vaccination does provide substantial protection against the more severe miliary and meningeal forms of this disease in children (96). On the other hand, the level of protection observed in adults has been low to non-existent, although there may be circumstances in which its use in healthcare workers and other high risk groups likely to be exposed to infection with drug-resistant tubercle bacilli should still be seriously considered (97). When assessing the level of protection achieved in many of these trials, it should be remembered that the intradermal vaccination procedure was developed to induce maximum levels of protection with a minimum of local discomfit, or adverse side effects (98). In fact, few attempts have been made to compare different routes and methods of inoculation (oral, intranasal, aerogenic, intradermal) for their relative immunizing efficiency in human volunteers. Development of improved vaccination methods will be essential if we are to bring this worldwide tuberculosis epidemic under effective control in the foreseeable future (99).

The exciting advances in our understanding of the molecular biology of the tubercle bacillus hold the promise of better diagnostics, more effective therapeutic reagents and improved vaccines (89, 100, 101). However, all of these products must be tested for safety and biological activity using quality control methods which have changed little in many decades. Part of the problem with vaccine testing has been the lack of a clear consensus regarding which surrogates best predict their protective activity in people. Tuberculin conversion rates have traditionally been used as a measure of BCG efficacy, but this parameter does not always agree with the level of protection achieved in the field (94). In an attempt to get around this problem, the presence of a number of cytokines such as IL-2, IL-12 and IFN-γ have been promoted as suitable predictors of a protective response in the vaccinated host (53). However, the relevance of these mouse cytokine responses to the level of protection likely to be achieved in a human population is not at all clear and it may still be necessary to subject these novel vaccines to extensive human field trial , despite the extravagant cost and protracted time that entails.

Laboratory assessment of a new anti-tuberculous vaccine seems deceptively simple in theory, but has proven to be far more difficult and contentious to achieve in practice (102). The most obvious factor will be the animal test system (103) but the vaccinating regimen will be important (size, route, number of inoculations, time to challenge, size and route of challenge), as well as the means used to assess the resulting immune response (increased survival time, reduction in bacterial growth in the lung, the nature and persistence of the T-cell response and its cytokine profile). In fact, as many as 20,000 variables are involved in a fully comprehensive assay of a new vaccine (102). Given such variability in the design of the test protocol, it is hardly surprising that widely differing results have been reported when the same vaccine was tested in different laboratories (104).

The National Institutes of Health has recently developed a standardized protocol for testing candidate anti-tuberculosis vaccines which are submitted for testing. Protection tests are first carried out in C57BL/6 inbred mice, then progress to similar studies carried out in guinea pigs. Some vaccines may be tested further in rabbits or monkeys depending on the intended indication for the vaccine. The route, dose, number of inoculations and time between doses can be varied somewhat to accommodate the manufacturer's specifications, but all animals will be subjected to a small aerogenic challenge with virulent M. *tuberculosis* Erdman (76). Protection can be assessed in terms of a significant

reduction in the number of viable Mycobacteria recovered from the lungs of the vaccinated vs. unvaccinated controls some 4 weeks after challenge. In addition, the spread of the infection to the spleen, the extent of granuloma formation within the lung and the intralesional cytokine profiles can be measured. Finally, the mean survival times for the vaccinated vs. control animals can be compared as a measure of long term protection. This testing protocol is both time consuming and labor intensive, but it provides a quantitative measure of the bactericidal or bacteriostatic activity developed by the vaccinated host compared to that achieved in a group of BCG vaccinated controls.

7. PERSPECTIVES IN PATHOGENESIS

It is a humbling thought that despite nearly a century of study, we still know so little about the ways that this pathogen antagonizes or neutralizes the normally efficient cellular defenses of the immunocompetent host. This lack is partly because we know very little about the mechanisms used by the normal host to block the development of clinical disease in most normal individuals exposed to this disease. We badly need to develop a better experimental model of latency in humans and the factors responsible for reactivational disease later in life. We do know that a number of genetic, nutritional, occupational factors are involved in this process (105, 106), along with physical and mental stress, alcoholism, silicosis as well as intercurrent parasitic, viral or bacterial infections (56, 107). Some strains of tubercle bacilli are clearly more infectious (pathogenic) than others, although the nature of the factors responsible for this variability is still unclear (16, 108). In fact, we know very little about which virulence gene(s) are responsible for the development of progressive disease, although it is generally assumed that a number of different factors which can neutralize the bactericidal activity of the activated macrophage are involved. However, the actual mechanism(s) involved in this complex process are largely unknown.

At the same time we know relatively little about the bacterial factors which are ultimately responsible for the death of the tuberculous host. The mycobacterial cell wall consists of a complex matrix of lipids, glycolipids and peptidoglycans many of which are responsible for the strongly adjuvantive properties of these organisms (63). While a number of products such as Wax D, cord factor and some sulfatides are toxic when injected in purified form into mice, it is not clear whether they are responsible for the symptomatology of the disease (65). The extensive immunochemical investigations carried out on the cell walls of this organism over the past decade have provided a clearer picture of the surface structures and the myriad of antigens associated with these complex organisms. The problem for the vaccinologist is to select the most protective of these antigens from an embarrassment of riches (109) and then to show that the purified antigen induces a fully protective immunity in an appropriately vaccinated host (76). Some of these antigens may act as effective immunogens in the mouse but not in the guinea pig and *vice versa*, and neither may serve as adequate predictors of the activity of the product in humans. At present, few of these purified antigens appear to be as effective as live BCG vaccine, which still remains the gold standard against which all immunizing preparations must be measured. Ideally, the vaccine of choice would prevent, rather than merely slow the growth of the challenge organism within the lung, but this type of response would be difficult to demonstrate experimentally given the limitations inherent in any currently available method for the quantitation of small numbers of Mycobacteria within the tissue. However, with the development of more powerful analytical tools it may be possible to detect the presence of the molecular mediators of a protective response during the critically important early phases of the host-parasite interaction. Development of these advanced techniques may even allow us to finally answer some of the questions posed more than a century ago by a country doctor armed with little more than a microscope, a wealth of pathological material and a keen, enquiring mind (110).

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