BCG vaccination and the tuberculin skin test in a country with low prevalence of tuberculosis

Epidemiological and immunological studies in healthy subjects

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To Åsa

&

To my mother

&

In memory of my father

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ABSTRACT

The immune response induced by vaccination with *Bacille Calmette-Guérin* (BCG) is not fully understood, and the interpretation of the tuberculin skin test (TST) is still under debate. This thesis was based on questions raised while implementing protective measures for healthcare workers and others at risk of exposure to tuberculosis (TB) in Sweden, a country where the prevalence of TB is low.

The present distribution of TST reactions in healthy young adults was analyzed, as well as the influence of various background factors on TST reactivity. Forty-two percent of BCG-vaccinated subjects had TST reactions ≥10 mm, while most unvaccinated subjects were non-reactive. BCG vaccination, geographic origin and age had decisive influence on TST reactivity. Most TST reactions in unvaccinated Swedish subjects were probably caused by cross-reactivity with non-tuberculous mycobacteria. Furthermore, the scar rate and TST reactivity after BCG vaccination was analyzed in children and adults. Vaccination of adults resulted in consistent scar formation, while scar prevalence in previously vaccinated children was low. There was a positive correlation between scar presence and TST reactivity in children as well as adults. Vaccinated subjects without a scar were TST positive more frequently than those non-vaccinated, indicating a systemic vaccine reaction in the absence of a local reaction.

New opportunities to elucidate the above-mentioned issues have evolved from insights in the immunology of TB. A T-helper 1 (Th1) response is known to confer protection against TB. Markers of a Th1 response are e.g. production of interferon-gamma and lymphocyte prolipheration after *in vitro* stimulation of peripheral blood mononuclear cells with tuberculin. These immune correlates were analyzed in relation to TST reactivity in previously BCG-vaccinated healthcare workers without known exposure to TB. Subjects with large positive TST reactions mounted a stronger Th1 response than TST negative subjects. Moreover, the corresponding *in vitro* analyses were performed before and after BCG vaccination of TST negative young adults. Both primary vaccination and revaccination caused a significant increase of the Th1 response, suggesting a protective effect against TB.

In conclusion, a history of BCG vaccination and/or the presence of a BCG scar are strong predictors of TST reactivity in our setting. A BCG scar can be used as an indicator of a technically correct vaccination in adults but does not have the same implication after vaccination of children. IFN-γ has a decisive role in the Th1 response and in resistance against TB, but protective immunity against TB is more complex than the effects of T cell derived IFN-γ production only.

The in vitro results should therefore be evaluated with caution. Yet, TST reactivity was associated with a protective immune response *in vitro* in BCG-vaccinated adults without known TB exposure, and a corresponding response was induced by primary vaccination as well as revaccination of young adults.

LIST OF PUBLICATIONS AND PRESENTATION OF THE THESIS

This thesis includes the papers listed below and a review concerning BCG vaccination, the tuberculin skin test and some epidemiological and immunological aspects of tuberculosis. Methods used in the four papers are described in the review, and the results are related to findings in the literature. The papers are referred to by roman numerals I-IV. The review is followed by a presentation of the aims of the thesis, brief summaries of the studies, and a short discussion of main results and key issues of the thesis.

I. Tuberculin skin test reactivity of young adults in a country with low prevalence of tuberculosis. Fjällbrant H, Rutqvist A, Widström O, Zetterberg G, Ridell M, Larsson LO. (In manuscript)

II. BCG scar and tuberculin reactivity in children and adults. Fjällbrant H, Ridell M, Larsson LO. Scand J Infect Dis 2008;40:387-392. (Reproduced with permission from the editor)

III. The tuberculin skin test in relation to immunological in vitro reactions in BCG-vaccinated healthcare workers. Fjällbrant H, Ridell M, Larsson LO. Eur Respir J 2001;18:376-380. (Reproduced with permission from the editor)

IV. Primary vaccination and revaccination of young adults with BCG: a study using immunological markers. Fjällbrant H, Ridell M, Larsson LO. Scand J Infect Dis 2007;39:792-798. (Reproduced with permission from the editor)

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
BCG	*Bacille Calmette-Guérin*
CD	cluster of differentiation
CFP-10	10 kDa culture filtrate protein
DTH	delayed-type hypersensitivity
ELIspot	enzyme-linked immunospot assay
ESAT-6	6 kDa early secretory antigenic target
HCW	healthcare workers
HIV	human immunodeficiency virus
IFN-γ	interferon-gamma
IGRA	IFN-γ release assay
IL	interleukin
kDa	kilodalton
LTBI	latent tuberculous infection
M.	Mycobacterium
MDR-TB	multidrug-resistant tuberculosis
MRC	Medical Research Council
NTM	non-tuberculous mycobacteria
OT	old tuberculin
PPD	purified protein derivative
PPD-B	PPD-Battey
PPD-S	PPD Standard
QFT	QantiFERON-TB Gold In-Tube
RD1	Region of Difference 1
SCID	severe combined immunodeficiency
TB	tuberculosis
Th	T-helper
TNF-α	tumor necrosis factor alfa
TST	tuberculin skin test
TU	tuberculin units
WHO	World Health Organization
XDR-TB	extensively drug-resistant tuberculosis

INTRODUCTION

After his grand discovery of *Mycobacterium tuberculosis* in 1882, the German microbiologist Robert Koch produced a liquid from culture filtrates of tubercle bacilli and used it for treatment against tuberculosis (TB) by subcutaneous injection (1). Unfortunately, his hopes for a remedy for the disease would soon be crushed. However, the potential of his liquid, "Tuberculin", as a diagnostic agent was discovered by Clemens von Pirquet (2), and in 1910 Charles Mantoux introduced the intradermal tuberculin skin test (TST) (3). This test could discriminate between subjects infected with *M. tuberculosis* and non-infected subjects.

It was apparent from many years of experience that people who remained healthy after infection with tubercle bacilli were relatively resistant to TB on re-exposure. The corresponding conclusion was drawn from studies of animals with healed tuberculous lesions that were challenged with tubercle bacilli. These observations inspired attempts to produce a non-virulent strain of the tubercle bacillus that would be capable of inducing protection against TB, without conferring risk of the disease. Albert Calmette and Camille Guérin of the French Pasteur institute finally succeeded in 1921, after a 13-year long attenuation process of a bovine strain of the tubercle bacillus (*M. bovis*). The strain was sub-cultured for 231 serial passages in a medium consisting of beef bile, potato and glycerine, while it gradually lost its virulence (4). The new non-virulent strain was designated *Bacille Calmette-Guérin* (BCG) and was originally given orally. The presently used intradermal route was developed in Göteborg by Professor Arvid Wallgren, starting in 1927 (5).

Two major principles of BCG vaccination adopted by Professor Wallgren were to only vaccinate TST negative subjects and that the immunizing effect was to be confirmed by a positive TST. His intention was to achieve a vaccination procedure analogous to the events of natural infection with tubercle bacilli. (The term "infection" in this review refers to infection without current signs or symptoms of disease.). It was observed that healthy subjects with a positive TST due to tuberculous infection were at reduced risk of developing TB from subsequent exposure compared with subjects who were TST negative. Subsequently, these observations were confirmed in several studies of healthcare students and healthcare workers (HCW) (6, 7). Although a corresponding association between resistance against TB and TST positivity induced by BCG vaccination was demonstrated by a study from Norway (8), the results of subsequent BCG trials have not supported this finding (9, 10). Consequently, the value of the TST as a correlate of protective immunity has been a subject of debate for many years.

The role of BCG in healthcare programs is also a subject of debate. There is no doubt of a relatively high efficacy of BCG vaccination in e.g. Scandinavia (8, 11-14), Great Britain (15), Northern United States (16, 17) and Canada (18), but the overall impression of the many vaccine trials in different parts of the world is one of variable and often contradictory results (19). BCG confers a high degree of protection against severe disseminated forms of TB in children (20), but a variable and incomplete effect against pulmonary TB in adults (21), the disease manifestation that propels the TB epidemic. In addition, waning of protective efficacy has been demonstrated in several BCG trials (22). These inadequacies of BCG have impelled a quest for new TB vaccines (23) as well as the practice of repeated BCG vaccination in many countries (24, 25).

Health authorities have the obligation to safeguard HCW and other professionals at risk of exposure to contagious TB. A wide range of alternative strategies are employed in different countries (26, 27). In Sweden, the choice has been selective BCG vaccination of students and professionals at risk, in addition to other occupational safety control measures. A TST is generally performed before the decision of BCG vaccination. The interpretation of the TST in this situation is complex, as well as the question of who will benefit from immunization with BCG.

New opportunities to evaluate protective immunity have evolved from insights in the immunology of TB. There is a continuous search for accurate correlates of immune protection (28, 29), which can be employed in the evaluation of new vaccines. Such immune correlates may also shed some light on the many questions involved in evaluating the immune status by the TST and in deciding on BCG vaccination.

This review discusses the issues regarding interpretation of the TST and the value of BCG vaccination in subjects at risk of TB exposure in a low-endemic setting. The focus is on evaluations of the immune response against tuberculin and BCG. The perspective is epidemiological as well as clinical and practical.

TUBERCULOSIS

GLOBAL EPIDEMIOLOGY OF TUBERCULOSIS

In 1993, the World Health Organization (WHO) declared TB a global emergency. During the following years, the TB epidemic in many parts of the world continued to increase. The total number of new TB cases is still rising due to population growth, but the global TB incidence rate, which peaked in 2004, leveled off during 2005 and 2006. It is estimated that 1.5 million people died from TB in 2006 and 9.2 million new cases were diagnosed (30).

TB is a leading cause of death in developing countries, in which approximately 95% of all new cases of TB and 98% of deaths occur. Although the highest rates per capita are in Africa, half of all new cases occur in six Asian countries (India, China, Indonesia, Pakistan, Bangladesh and the Philippines). In developing countries TB affects mostly young adults in their most productive years, thereby contributing to the unfavorable socio-economic development in many areas.

According to a recent estimate, nearly one third of the world's population is infected with tubercle bacilli (31). Co-infection with human immunodeficiency virus (HIV) multiplies the risk of progression to disease (32) and is therefore an important contributor to the global TB epidemic. The association with HIV is especially strong in sub-Saharan Africa, where rates of HIV infection among TB patients exceed 50% in several countries (33). The impact of the "cursed duet" of TB and HIV on the welfare of this region has been devastating.

Drug-resistant strains are an increasing problem, emerging from the misuse of TB drugs (34). Multidrug-resistant TB (MDR-TB), resistant to the key drugs of the standard treatment regimen, was seen in 5% of the cases in 2006 (35), with the highest rates in countries of the former Soviet Union and China. Treatment of MDR-TB is protracted, costly, poorly tolerated and less effective than treatment of non-resistant strains (36). Cases with resistance also to the major second-line drugs are denoted extensively drug-resistant TB (XDR-TB) and have recently emerged in all regions of the world (35). XDR-TB is extremely difficult to treat (37) and threatens to derail the recent progress in TB control.

EPIDEMIOLOGY OF TUBERCULOSIS IN EUROPE AND SWEDEN

The incidence of TB in industrial countries decreased rapidly during the 20th century (38). In many European countries the decline has halted during recent years, and some countries have seen an increase of TB rates. The development of the TB epidemic in Sweden parallels these trends. After a century of rapid decline, the number of TB cases in Sweden leveled off during the 1990's to a rate between 400 - 500 cases per year, corresponding to an incidence of approximately 5 per 100 000 (39). As in many other low-endemic countries, most TB cases occur in young immigrants and elderly native-born individuals. The incidence of TB among immigrants reflects the incidence of their native country (40, 41), and remains high several years after arrival (42, 43). Immigrants constitute an increasing part of the Swedish TB cases (78% of the TB cases in 2007), thereby counteracting the continued decline in the Sweden-born population.

Despite one of the lowest TB rates in the world, the Swedish TB program has obvious problems. The last three years have involved a marked increase in the TB incidence (39). A large cluster of isoniazid-resistant TB has been reported (44), indicating ongoing transmission among immigrants. The incidence of resistant and multi-resistant strains is increasing (45), the rates of completed treatment have been low and contact-tracing inadequate (44). In addition, the favorable situation with very little TB naturally leads to an unawareness of the diagnosis of TB. Consequently, outbreaks have occurred due to prolonged doctor's delay, e.g. affecting non-vaccinated children at day-care centers (46).

BACTERIOLOGY

TB is caused by bacteria of the *Mycobacterium tuberculosis* complex (47), which includes the major pathogen *M. tuberculosis*, as well as *M. africanum* (48, 49), *M. bovis* (50), *M. bovis BCG*, *M. canettii* (51), *M. caprae* (52), *M. microti* (53) and *M. pinnipedii* (54). Other members of the genus *mycobacterium* (55) are *M. leprae,* the causative agent of leprosy, and the large group of non-tuberculous mycobacteria (NTM).

Mycobacteria are acid-fast rod-shaped bacteria, 2-5 μm long. All *Mycobacterium* species share a characteristic lipid-rich cell wall, thicker than in many other bacteria, composed of mycolic acids, complex waxes, and unique glycolipids. The unusual cell wall structure endows mycobacteria with resistance to dehydration, acids and alkalis and most antibiotics. The cell wall also helps mycobacterial pathogens to survive within macrophages (56).

M. tuberculosis is a strict aerobe with a slow growth rate. The doubling time is 12-24 hours in vitro as well as in vivo. Consequently, identifiable mycobacterial colonies may not appear for 4 to 6 weeks on solid media.

NTM were previously denoted atypical mycobacteria, since they were observed as rare and divergent findings in mycobacterial cultures in which *M. tuberculosis* was the dominant species. As the multitude of NTM species were discovered, it became clear that “atypical” was a better term for the species of the *M. tuberculosis* complex: they are unique among mycobacteria as obligate parasites that survive only in humans or animals, in which they often cause disease, while the abundance of NTM are low-virulent opportunists ubiquitous to the environment. Thus, an alternative and more descriptive label for NTM is "environmental mycobacteria". Common habitats are natural waters, drinking water and soil (57). Currently, more than 125 NTM species have been identified (58).

TUBERCULOSIS TRANSMISSION AND HOST DEFENSE

The major route of TB transmission is by inhalation of tubercle bacilli from aerosols, expectorated by individuals with TB in the airways when they cough, sneeze, talk or sing (59, 60). The smaller particles in the aerosols are rapidly dehydrated, forming tiny droplet nuclei (about 5 μm in diameter) which may remain airborne for many hours. When inhaled the droplet nuclei are sufficiently small to reach the distal airways, whereas larger particles are deposited on the walls of more proximal airways and cleared by the mucociliary apparatus. The less common route of infection with tubercle bacilli is by ingestion. In areas where dairy products are not properly treated and bovine TB has not been eliminated, ingested *M. bovis* organisms may cause direct infection of the gastrointestinal tract.

In the alveoli the bacilli are phagocytosed by macrophages. Tubercle bacilli have the ability to survive and even multiply within macrophages through evasive strategies that are not clearly understood (61). Depending on the capacity of the host’s innate resistance, to which e.g. natural killer cells and neutrophils are also believed to contribute, the bacilli can be killed in a process leading to apoptosis – a programmed series of events intrinsic to all cells that leads to cell death without causing inflammation and tissue destruction. Establishment of infection and further immune events may thereby be prevented (23). Inhibition of apoptosis has been suggested as a central strategy of tubercle bacilli for intracellular survival (62, 63).

If the bacilli are able to survive initial defenses, intracellular proliferation may cause cellular necrosis and release of the organisms. Subsequent production of chemokines and cytokines attract other immune-effector cells which engulf the bacilli, resulting in further intracellular growth, necrosis, inflammation and local spread of the infection. In this ongoing pathological process, tubercle bacilli are transported to regional lymph nodes by dendritic cells - a subset of phagocytic cells specialized in activating naïve lymphocytes after migrating from the infectious site (64, 65). Processed peptide antigens from the bacillus are presented in conjunction with major histocompatibility complex molecules on the surface of dendritic cells, allowing interaction with receptors of naïve T cells (66). Following antigen encounter the T cells undergo rapid proliferation and differentiate into effector cells (67), that subsequently migrate to the site of infection.

The activation of T cells in the lymph nodes normally takes place within 3-8 weeks after infection. Activated T cells are the core of the specific cell-mediated immunity that eventually can limit multiplication of bacilli and spread of the infection. In parallel, delayed-type hypersensitivity (DTH) develops against tuberculous antigens (see next page), as illustrated by a positive TST (64).

At the site of infection, activated T cells interact with infected macrophages. Interleukin (IL) -2, IL-12 and IL-18 released by the macrophages induce T cell production of interferon-gamma (IFN-γ), the key cytokine in the protective immune response (61, 68) with decisive influence on the further events of cell-mediated immunity. IFN-γ stimulates the phagocytosis of tubercle bacilli within the macrophage, thereby converting the macrophage from immunologically naïve to a specifically immunocompetent effector cell. In addition, IFN-γ stimulates the macrophage to release tumor necrosis factor alfa (TNF-α)**,** which promotes the formation of granulomas by T cells and macrophages. The ability of the granulomas to control the spread of the bacilli determines the fate of the infection. The tubercle bacilli are mainly contained in the characteristically necrotic centre of the granulomas, thereby limiting further replication and spread of the organism (69, 70). The crucial role of TNF-α in this process is illustrated by the rapid reactivation of TB in treatment with TNF-α-blocking agents (71).

Unlike many other pathogenic bacteria, which contain endotoxins and exotoxins, the pathologic effects of tubercle bacilli are largely mediated by the immune response of the host. There is a complex balance between control of infection and tissue destruction in TB. According to findings in mice, this balance is dependent on the type of T cell response against the infection. T-helper 1 (Th1) responses are characterized by the production of IFN-γ, IL-2 and

Interleukin-12 and are considered to be required for protection against intracellular infections (72). T-helper 2 (Th2) responses, characterized by the production of IL-4, IL-5, IL-10 and IL-13, protect against e.g. helminth infections and are involved in atopic reactions (72). In humans, TB is characterized by decreased levels of Th1 cytokines compared to the levels in subjects with latent TB who are capable of controlling the infection (73-75), and it is clear that a Th1-response is a crucial component of human protective immunity against TB (61). Results regarding Th2 cytokines in TB patients have been conflicting (75, 76). However, recent studies indicate that previous inability to demonstrate IL-4 in human disease may have been due to technical difficulties (77), and that IL-4 may have an important role in the pathogenesis of human TB. Rook and colleagues suggest that the production of IL-4 superimposed on Thl activity can convert the response from protective to pathological (77) (see p. 59). The competitive inhibitor of IL-4, IL-4δ2 (78, 79), was increased in healthy individuals with LTBI (73), suggesting that long-term control of LTBI is associated with inhibition of the Th2 response. According to this theory, disease progression involves a shift form Th1 to Th2, with increased IL-4 activity and a decrease in IL-4δ2 (77).

Views regarding the significance of DTH for resistance against disease are divergent (see p. 64). DTH is a Th1 response that involves cytotoxic mechanisms leading to the killing of infected macrophages (80). The detrimental effects of DTH in the lungs develop if large amounts of tubercle bacilli are present (81). When many bacilli accumulate within the macrophages, the cytotoxic response kills not only the infected macrophages but also some of the surrounding tissue, thereby forming the caseous center of the granuloma. When bacilli escape from the edge of the caseum, they are ingested by nearby macrophages. If these macrophages do not control growth of the bacilli, the cytotoxic immune response again kills the bacilli-laden macrophages (and surrounding tissue), thus enlarging the caseous center. In hosts that develop poor activation of macrophages, this process may occur repeatedly and lead to extensive tissue destruction. DTH is the principle mechanism behind tissue destruction in TB, but without DTH the control of bacillary growth would be reduced (80).

Antibody responses are considered to contribute little to protection against TB. However, mycobacterium-specific antibodies may be capable of enhancing both innate and cell-mediated immune responses (80). In a recent study, BCG-induced antibodies improved phagocytosis by macrophages and increased proliferation and IFN-γ production of mycobacterium-specific T cells (82).

When the infection is not properly contained, bacilli may spread systemically from the primary lesion and regional lymph nodes to multiple organs. In some

individuals, proliferation of bacilli continues until the infection becomes severe enough to cause disease - so called primary TB. However, in the majority of infected subjects, cell-mediated immunity is effective enough for the infection to subside to a state in which tubercle bacilli remain dormant within the infectious foci. In this form of latent TB, viability of the bacilli is maintained and reactivation may occur later in life. A small number of antigen-specific T cells survive and become long-lived memory T cells (83).

Progressive, uninterrupted invasion by tubercle bacilli occurs mainly in infants, small children and immunocompromised individuals, particularly in those with HIV infection. Manifestations of primary TB are meningitis, miliary disease and pleuritis, as well as primary progressive forms of pulmonary and lympho-glandular TB.

Risk factors for reactivated disease are HIV infection, diabetes mellitus, end-stage renal disease, silicosis, certain malignancies, malnutrition, old age and immunosuppressive treatment. Individuals with apical fibronodular scarring of the lungs after previous (generally subclinical) TB are at particular risk. The lungs, lymph nodes and bones are the most common sites of reactivated disease.

OCCUPATIONAL RISK OF TUBERCULOSIS IN HEALTHCARE WORKERS

The risk of tuberculous infection and disease is generally considered to be higher among HCWs than in the general population. Studies of HCWs in developing countries demonstrate a substantially increased risk (96). However, in high-income countries the risk compared to the surrounding community is variable (97, 98). A recent review found that the occupational risk for HCWs of high-income countries can be considerable in facilities with many TB patients, particularly if the infection control measures are inadequate (98). Casual contacts with patients in healthcare settings involve a relatively low risk of TB transmission (99), whereas the risk is substantial in connection with autopsy and TB laboratory work (100), as well as in aerosol-generating procedures such as bronchoscopy, intubation, suctioning of the airways and sputum induction. Furthermore, the risk of nosocomial transmission of TB is augmented by an increasing proportion of immigrants and a rising prevalence of HIV infection and drug-resistance.

Prevention of TB transmission in health care settings (99, 101) include a hierarchy of three strategies, of which *administrative measures* are considered crucial, *engineering measures* valuable and *personal respiratory protection* possibly effective under certain circumstances (27, 98, 102). Administrative

measures refer to actions promoting e.g. early diagnosis and efficient treatment, engineering measures principally involve adequate isolation of contagious patients, and personal respiratory protection refers to the use of mask respirators.

Protection of HCW by BCG vaccination is an additional component of the administrative strategy that has sparked intense debate during the years (103-115). Several controlled studies in HCWs have reported a protective effect for BCG vaccination (reviewed in (26)). However, due to the induction of TST reactivity, the BCG strategy is in conflict with the alternative measure of periodic tuberculin skin testing, which aims at treating latent TB in subjects with TST conversion (101) (see p. 37). The TST program has been widely practiced in the United States, emphasizing the possibility of surveillance as a major advantage (116). In other low-endemic countries BCG vaccination is recommended (117, 118) in agreement with the principle of optimizing individual protection of individuals at increased risk of exposure. Both sides of the debate address well-known shortcomings of the opponents' strategy.

Decision analyses comparing BCG vaccination and periodic tuberculin skin testing of HCWs in the United States have favored the use of BCG (107, 108, 111), even assuming low levels of BCG effectiveness. However, these conclusions have been vigorously debated by Reichman and colleagues (113, 115, 119). Lately, the emergence of MDR-TB has renewed interest in the BCG strategy (106, 112, 120), since treatment of latent forms of MDR-TB is insufficiently documented and may be complicated (36). Furthermore, a recent study suggests that longitudinal TST studies are valuable for surveillance of the occupational risk of TB even in BCG-vaccinated populations (121).

NON-TUBERCULOUS MYCOBACTERIAL INFECTION AND DISEASE

Natural and indoor water sources are considered the primary reservoir for most human NTM infections (57). Transmission occurs either through inhalation or ingestion. There is no evidence of human-to-human transmission of NTM (58). Infections with NTM are common in populations where the bacilli are abundant in the surroundings (84-87), but latent NTM infections have not been observed (88). An increase in infections (89) as well as in NTM disease (58, 90-92) during the latter part of the 20th century has been reported. In Sweden, infections with NTM are common in children (93), and the incidence in children of lymph node lesions and soft tissue lesions appear to have increased after the general BCG-vaccination of newborns was discontinued (94).

Pathogenesis and host defense mechanisms of NTM disease are similar to TB (95). The most common clinical manifestation of NTM in industrialized countries is lung disease similar to TB in middle-aged and older individuals. Other important manifestations are cervical lymphadenitis in small children, skin/soft tissue diseases, and disseminated disease in immunocompromised hosts (58).

THE TUBERCULIN SKIN TEST

TUBERCULIN PRODUCTS AND THEIR STANDARDIZATION

The first tuberculin was prepared by Robert Koch by filtration of heat-sterilized cultures of *M. tuberculosis* grown on veal broth, followed by evaporation of the filtrate to 10% of its original volume (1). This type of tuberculin contained remains of veal broth and therefore frequently induced non-specific reactions. Replacing the veal broth with a synthetic culture medium improved specificity. Such products are called Old Tuberculin (OT). In the 1930's, Florence Seibert developed a technique of precipitation with ammonium sulphate to isolate proteins from autoclaved culture filtrates of tubercle bacilli. Results with this new type of tuberculin denoted Purified Protein Derivative (PPD) proved more reproducible and specific than OT. In spite of the designation "purified protein derivate", polysaccharides are present in addition to proteins, even in modern PPD products (122). Heat-sterilization coagulates much of the culture proteins, leaving relatively small proteins with a molecular weight in the range of 10 kDa (123-125). The small size of the proteins explains why PPD is not immunogenic, i.e. that a TST does not induce hypersensitivity to PPD on following tests in individuals previously non-sensitized to mycobacteria (122, 126).

After careful standardization, a large batch of PPD was eventually produced by Seibert in 1939, termed PPD Standard (PPD-S) (127). In 1952 a portion of this batch was adopted as an international standard by the WHO. Even today, all other PPD:s should be standardized against this product.

On request from the United Nations International Children's Emergency Fund (UNICEF) a large batch of tuberculin PPD was produced by Statens Serum Institut in Copenhagen, which was taken into use in 1958. In line with previous PPD products from Statens Serum Institut, the new batch designated PPD RT23 was precipitated by trichloracetic acid. Its total dry weight was 670g, theoretically corresponding to approximately 17 billion tests. The purpose of such a large batch was to meet global demands for an extended time, thereby improving comparability of TST data. PPD RT 23 is still used today worldwide, and the supply will continue to fulfill the demands for the foreseeable future (Hasløv K, personal communication).

Operating characteristics of a diagnostic test

Diagnostic accuracy

The *sensitivity* of a test is the percentage of people with a given condition who have a positive result ("true positives"). If false negative results are uncommon, the sensitivity is high. The *specificity* of a test is the percentage of people without a given condition who have a negative result ("true negatives"). False positive results decrease the specificity of a test.

Predictive ability

The predictive value of a positive test result (*the positive predictive value*) is the percentage of positive results that correctly identifies the presence of a given condition. *The negative predictive value* is the percentage of negative results that correctly excludes the presence of the condition.

Influence of prevalence

The sensitivity is only associated with individuals having the condition, whereas the specificity exclusively deals with individuals without the condition. Consequently, these test qualities are not affected by the prevalence of the condition in the population. In contrast, the positive and negative predictive values are dependent on the prevalence of the condition; with increasing prevalence the positive predictive value is enhanced (as the rate of true positive results increases) and the negative predictive value is reduced (as the rate of true negative results decreases).

Doses of PPD:s are for practical purposes expressed in Tuberculin Units (TU). 1TU is defined as a specified amount of the dry substance of protein (0.02 μg for PPD-S as well as for PPD RT23). The optimal dosage of PPD-S was determined by testing individuals with high as well as low likelihood of tuberculous infection with increasing doses (128). A dose of 5 TU caused a positive reaction in nearly all TB patients and many TB-exposed contacts, whereas increasing doses did not evoke more positive reactions. In contrast, reactivity in unexposed subjects was low and increased slightly with increasing doses up to 5 TU, whereas higher doses sharply enhanced reactivity.

Consequently, 5 TU of PPD-S was the best compromise between sensitivity and specificity and became the recommended standard dose.

During the efforts to standardize PPD RT23 against PPD-S (129), it became obvious that it was impossible to define doses that were equipotent in all situations; the potency ratios differed with the type and level of TST sensitivity in the populations tested. Since the primary purpose of tuberculin skin testing is to measure the prevalence of tuberculous infection, priority was given to populations with sensitivity assumed to be mainly caused by such infections. In a subsequent survey of TB patients and non-vaccinated recruits in the United States, the potency of 2 TU of PPD RT23 was relatively equipotent to 5 TU of PPD-S (130), i.e. the sensitivity was similar. However, in the US survey as well as in the standardization studies (129), the specificity of PPD RT23 was markedly lower, with considerably larger reactions than PPD-S in populations with high rates of NTM infections. The reactions were also larger to PPD RT23 in BCG-vaccinated populations according to the standardization studies (129).

In spite of these differences, 2 TU of PPD RT23 has eventually become generally accepted as an approximate equivalent of the 5 TU dose of PPD-S and is now recommended by WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD) for skin test surveys (131). However, in e.g. India, the dose of 1 TU of PPD RT23 is recommended, due to its observed higher specificity and equal sensitivity in national surveys (132).

TST surveys in South Korea have questioned whether PPD RT23 has lost potency over time (133). In response, Statens Serum Institut has published its quality control data, indicating no decline in potency, but rather pointing to local problems in the dilution or other handling of PPD RT23 (134). Additional recent studies indicate that the potency of PPD RT23 is preserved (132, 134-136).

SENSITINS

Sensitins are antigen preparations from culture filtrates of mycobacteria mainly used for skin testing and capable of eliciting DTH reactions in hosts sensitized to mycobacteria of the same or related species. In other words, tuberculins are sensitins. However, the term sensitin is generally used only for preparations derived from NTM.

Sensitins are produced from different species of NTM in the same way as PPD:s. Commonly used sensitins are PPD-B from *M. intracellulare* (the "Battey antigen"), *M. avium* sensitin RS10 and *M. scrofulaceum* sensitin RS95.

The two latter sensitins were produced by Statens Serum Institut until 2003. Although sensitins are PPD:s, the term PPD generally refers to tuberculin PPD, which is also how the term is used in the present paper.

Comparative skin testing with sensitin and tuberculin can be used to differentiate between infection due to NTM and tubercle bacilli. This method has been useful in epidemiological (86-88) as well as in clinical studies (137, 138). However, the diagnostic efficacy was less in other clinical studies (139, 140), and the clinical routine use of sensitins has been limited.

IMMUNE RESPONSE TO TUBERCULIN

An intradermal injection of tuberculin induces a DTH reaction in subjects previously sensitized to mycobacteria. DTH reactions, which also include contact hypersensitivity and granulomatous hypersensitivity, are characterized by a cell-mediated response with delayed onset, and reflect the presence of memory T cells (long-lived antigen-specific CD4 cells) which initiate the reaction.

The histological and immunological events of the TST reaction were recently reviewed by Vukmanovic-Stejic (64). After the injection of tuberculin, dendritic cells and Langerhans cells residing in the skin become activated through innate immune mechanisms and begin to phagocytose antigenic material. The subsequent cellular infiltration into the skin is biphasic: an early non-specific reaction dominated by neutrophils and monocytes is followed by a slower antigen-specific recruitment of T cells. Initially, macrophages are activated by IFN-γ to produce TNF-α and IL-1. These pro-inflammatory cytokines and chemokines act on endothelial cells in the capillaries to express adhesion molecules, which in turn bind to receptors of neutrophils and recruit them to the inoculation site. This non-specific reaction also occurs in unsensitized subjects. The influx of neutrophils begins within a few hours and is followed by an increasing infiltration of monocytes. Antigen presented by the resident innate immune cells lead to the activation of antigen-specific T cells, which begin to accumulate around dermal blood vessels after about 12 hours. Whether T cells are activated in the skin or in draining lymph nodes has not been established. After 24 hours the majority of infiltrating cells are macrophages, whereas T cells are in majority after 48 hours. The cellular infiltrate subsequently disrupts the collagen bundles of the dermis and expands the tissue. The peak of the DTH reaction occurs 48-72 hours after the tuberculin injection (141). The cellular infiltrate may then be palpable as an induration of the skin and is often accompanied by edema and erythema due to dilatation and congestion of the capillaries. Formation of vesicles and bullae indicates a high degree of

tuberculin sensitivity and the presence of tuberculous infection (142, 143). In such individuals the reaction may become severe enough to cause ulceration and necrosis at the test site (the Koch phenomenon).

TESTING TECHNIQUES

The two major techniques currently used for tuberculin skin testing are the Mantoux method and the multiple puncture method. However, only the Mantoux method is included in official recommendations (117, 131, 144).

The Mantoux method

The Mantoux method involves a strictly intradermal injection of an exact dose of tuberculin. The preferred site of injection is the volar or dorsal aspect of the mid third of the forearm. A standard 1 ml graduated tuberculin syringe fitted with a short bevel needle (gauge 25-27) is recommended. Injection of 0.1 ml of PPD solution should produce a wheal of 6 to 10 mm in diameter if the injection is done correctly. If a wheal does not appear, the solution has been injected too deeply, and the test should be repeated on the other arm or at least 4 cm from the first injection site.

The Mantoux test is read 48-72 h after injection by measuring the diameter of the induration in millimeters transversely to the long axis of the forearm. Standardization as well as information regarding the future risk of TB is based on TST reactions measured by this principle and at this time interval. Consequently, other time points of reading should be avoided, as well as other recordings of reaction size, such as the mean size of two induration diameters or the size of the erythema (145, 146).

Tuberculin skin testing demands considerable skill to be reliable and the medical personnel should be specially trained for the method. The intradermal injection is a particular challenge in small children, but the major difficulty is reading and measuring of the induration. Test reading by inexperienced readers, such as patients, is strongly discouraged.

The gold standard for measuring the induration is by palpation. The margins of the induration are found by drawing the index finger lightly across the reaction. The outer edges of the reaction are marked, and the induration is measured at its widest diameter with a flexible ruler. The standard deviation (the average variation of readings) of TSTs measured by the same experienced reader was 1.3 to 1.9 mm in one study (148). Inter-reader variability resulted in slightly

larger standard deviations of 2.3 - 2.5 mm (149, 150). An alternative to palpation is the ball-point pen method (151, 152).

Digit preference

TST readers have a tendency to round off induration measurements to predetermined cut-off values or ending digits such as 0, 5 or even numbers. This phenomenon is known as digit preference (153) and is often revealed when a quantity of TST readings are displayed in frequency distributions. This problem can result in substantial misclassifications (154) but may be minimized by use of measuring callipers (141). In addition, the distortion by digit preference of frequency distributions and statistical analyses can be corrected by simple (**I**) (155) as well as more advanced statistical methods (153).

The multiple puncture test and other testing techniques

A multiple puncture test (such as the Tine test and the Monotest) introduces tuberculin into the skin either by a device with points coated with dried tuberculin or by puncturing through a film of liquid tuberculin. The advantage of these tests is the speed and ease with which they can be administered, even by unskilled personnel. However, the quantity of tuberculin introduced into the skin cannot be precisely controlled, and the sensitivity, specificity and reproducibility of the tests are generally lower than for the Mantoux method (141).

Several other methods of skin testing have been used, e.g. the Heaf test (156), the Pirquet test (2, 157) and the Moro test (158).

APPLICATIONS OF THE TUBERCULIN SKIN TEST

The TST is often used in the diagnosis of active TB, but its main utility is in diagnosing latent tuberculous infection (LTBI). To increase the yield of TST activities, a targeted approach is recommended that identifies individuals with a high likelihood of LTBI and/or a high risk for progression to TB (159). The aim is to select high-risk subjects for preventive treatment or intensified surveillance. Several randomized trials have shown that treatment of LTBI, diagnosed by the TST, reduces the risk of TB by 60% to 90% (159). Situations in which the TST is utilized are mentioned below.

As an aid in the diagnosis of active TB

The TST is often used in the work-up of suspected TB patients. However, the effectiveness of the TST in this situation is limited by its relatively low sensitivity in TB patients (160-162) (see p. 29). Furthermore, the TST does not allow a distinction between disease and LTBI. The utility of the TST as a potential indicator of disease is therefore mainly restricted to populations where the prevalence of LTBI is low, as in children from low-endemic countries. Difficulties in attaining microbiological confirmation increase the supportive role of the TST in the diagnosis of TB, as in children and in patients with extra-pulmonary disease.

Contact tracing

The TST has a particular high yield in close contacts and constitutes an essential tool in the measures for TB prevention when treatment of LTBI is implemented in newly infected individuals. The likelihood of LTBI among close contacts of a contagious TB case is generally 30-50% (163). Newly acquired tuberculous infection is associated with a high risk of progression to active TB the first 1-2 years after exposure (164) (see p. 38). Furthermore, the rate of active TB among close contacts has been estimated to 1-3%, more than 100-fold higher than in the general population of low-endemic countries (163-166).

Regular surveillance of healthcare workers

Periodic tuberculin skin testing can be used for surveillance of TST negative individuals at risk for exposure to *M. tuberculosis*. Annual TSTs are widely used for surveillance of HCW in the United States (159).

Epidemiological surveys

TST surveys undertaken in groups of e.g. school children provide information from which the average annual risk of infection can be estimated (167). This parameter is considered a reliable indication of the level of LTBI in a community (38). Furthermore, the trend of infection over time may be determined by repeated surveys at regular intervals. These epidemiological methods are important tools in the planning and evaluation of national TB programs.

Selection of individuals for BCG vaccination

The results of pre-BCG vaccination TSTs may be used as a basis for selection of individuals eligible for BCG vaccination. Pre-vaccination TST reactivity is associated with a reduced protective efficacy of BCG (see p. 57) and it is generally agreed that TST positive individuals do not benefit from BCG vaccination (168). In addition, vaccination of TST positive individuals is associated with more discomfort and an intensified local reaction (see p. 48).

Control of BCG vaccines and BCG vaccination procedures

Tuberculin skin testing is used in the quality control of BCG vaccines (169). A proven ability to induce TST reactivity is generally required for a new BCG vaccine to be licensed. The TST is also used as a quality indicator of vaccination procedures: if the BCG vaccine is not handled properly in the field, it may lose its protective efficacy as well as its ability to induce TST reactivity (170, 171).

SENSITIVITY OF THE TUBERCULIN SKIN TEST - REACTIVITY IN INDIVIDUALS WITH ACTIVE OR LATENT TUBERCULOSIS

DTH to tuberculin usually develops 6-8 weeks after initial tuberculous infection (141). Although the sensitivity of the TST in a healthy young person is generally high, knowledge of the mechanisms behind false negative reactions is essential for correct interpretation of the test.

False negative reactions

It is commonly believed that DTH induced by tuberculous infection generally persists until old age (144). Reversion of TST reactivity is indeed common in the elderly (172, 173) but is also documented at lower rates in younger people (174, 175). The persistence depends on the infectious dose as well as on the extent of re-exposure to mycobacteria (174, 175). Many factors can diminish reactivity, from conditions that impair DTH (144) (see Table) to technical problems such as improper storage of the tuberculin reagent and errors in administration or reading.

Table. Conditions associated with diminished tuberculin skin test reactivity

Viral infections (HIV, measles, mumps, chicken pox)
Live virus vaccinations (measles, mumps, polio, varicella)
Disseminated TB (Miliary TB, TB meningitis), tuberculous pleurisy
Other extensive bacterial infections (typhoid fever, typhus, leprosy, pertussis)
Chronic renal failure
Malnutrition
Diseases of lymphoid organs (Hodgkin's disease, lymphoma, chronic leukemia, sarcoidosis)
Immunosuppressive treatment (corticosteroids, chemotherapy, TNF-α blockers)
Age (newborns, elderly)
Stress (surgery, burns)

An important factor to consider in non-reactive individuals is the possibility of anergy. Lymphocytes are said to be anergic when they fail to respond to their specific antigen. In cutaneous anergy, absence of DTH to an intradermal injection of tuberculin occurs in spite of the presence of tuberculous infection. Anergy can be associated with all the conditions mentioned in the above table and is generally an on-off phenomenon; the reaction is completely absent rather than decreased in size (141).

TST anergy has been described in immunocompetent individuals with pulmonary TB (160, 176) and may lead to limited granuloma formation and poor clinical outcomes in TB patients (177). Anergy is associated with defective T cell responses including an antigen-specific impaired ability to produce IL-2 and to proliferate in response to challenge with tuberculin (177). T cells from anergic patients produced IL-10 but not IFN-gamma and there is evidence that IL-10 mediates a direct anergizing effect on T cells (177).

Tuberculosis patients

The TST reactivity of TB patients has been studied in large international surveys using PPDs standardized to 5 TU of PPD-S (178). Patients with different forms of disease, of different races and ages, and from different countries produced reactions that formed remarkably uniform distributions, resembling the shape of a normal curve around a mode averaging 14-18 mm. Only few reactions measured <6 and >25 mm in these surveys.

It should be noted that the patients in the above-mentioned surveys were already on treatment. Studies of newly diagnosed TB patients have revealed higher rates of false negative reactions in the range of 15-50% (160-162, 179). In one of these studies reactivity was restored in most patients after two weeks of treatment (161). In a meta-analysis of 14 relatively small studies for evaluation of IFN-γ release assays (IGRAs, see p. 42), the pooled sensitivity of the TST was 71% (180).

Common conditions associated with reduced TST reactivity in TB patients are advanced disease (160, 181), malnutrition (182) and advanced age. Studies of elderly patients have shown false negative rates of up to 30% (172, 173). In an international perspective, HIV infection is a frequent cause of anergy (183). With the mentioned exceptions in mind, it can be concluded that young HIV-negative TB patients in good physical condition, without high or prolonged fever, will in most instances have a positive TST.

Latent tuberculous infection

When frequency distributions of TST reactions are compared between subjects with increasing likelihood of TB exposure, groups with the highest gradient of exposure show distribution modes corresponding to TB patients (184, 185). These findings indicate that TST reactivity in healthy individuals with tuberculous infection is no different from those in which the infection has progressed to disease. The same conclusion was drawn from a study of Alaskan Eskimos, among whom tuberculous infection was prevalent but exposure to NTM was rare (186). The data of healthy subjects showed a bimodal distribution of reactions with modes at 0 and 18 mm and only few reactions between 2 and 5 mm. The authors concluded that reactions of ≥5 mm were indicative of tuberculous infection. Other surveys of populations with corresponding mycobacterial exposure have showed similar normal distributions (178).

There is no readily applicable gold standard available for the diagnosis of latent TB. Consequently, the sensitivity (as well as the specificity) of the TST in diagnosing latent TB is impossible to ascertain. In the absence of a gold standard, newly diagnosed active TB is commonly used as a surrogate for latent TB to estimate sensitivity (180). However, this is a poor surrogate because of the known reduction in cell-mediated response in TB patients, particularly at the time of diagnosis. Patients undergoing treatment for active TB who have clinically recovered are at present the closest approximate to healthy subjects with known tuberculous infection. The above-mentioned WHO study from 1955 (178) mainly included such patients and showed a sensitivity of 98%. Furthermore, in three recent studies with corresponding patients, as well as

patients with completed TB treatment, the sensitivity was 95-96% (187). As in active TB, the expected sensitivity in populations with latent TB is reduced in immunocompromised subjects (see Table), such as in HIV infection (188, 189), chronic renal failure (190, 191) and hematological patients (192).

Several prospective cohort studies are currently being conducted in different settings to estimate the risk for progression to active disease in individuals who have undergone testing with the TST and IGRAs (193, 194). These studies are based on the current gold standard for the diagnosis of latent TB: the demonstration of subsequent development of TB. This method has a high specificity but an expected sensitivity of only about 5% (the expected disease rate the first years after infection), although those diagnosed are the clinically most relevant, i.e. those in need of treatment or close follow-up of their tuberculous infection. For the identification of subjects with an effective immune response to tuberculous infection, other methods are warranted, possibly similar to in vitro correlates of vaccine-derived protective immunity (see p. 66).

SPECIFICITY OF THE TUBERCULIN SKIN TEST - REACTIVITY IN INDIVIDUALS WITHOUT TUBERCULOUS INFECTION

Some antigens in tuberculin are shared with NTM (123, 124, 195, 196) as well as with BCG (197). A tuberculin injection in subjects with NTM infection or previous BCG vaccination can therefore cause skin indurations due to cross-reactivity **(I)** (198). The TST in BCG-vaccinated individuals will be discussed in detail below (p. 50). Cross-reactions in subjects with NTM infections are generally small (86, 184, 199). The overlap with reactions caused by tuberculous infection may nevertheless be considerable in areas where NTM are common in the environment **(I)** (93, 137, 199, 200). The larger the reaction size, the greater is the likelihood of tuberculous rather than non-tuberculous infection. Although a general maximum size limit for cross-reactions cannot be specified, NTM-induced TST reactions rarely reach the size of 15 mm (199, 201, 202).

False positive TST reactions due to cross-reactions with NTM and BCG result in a decreased specificity of the test. As mentioned above, the sensitivity of the TST in detecting tuberculous infection is well-standardized and relatively constant between different settings. In contrast, the specificity is less predictable and varies with the prevalence of BCG vaccination (198, 203, 204) and NTM infections (38, 141). In the absence of a gold standard for the diagnosis of latent TB, low-risk populations are used to estimate the specificity of the TST **(I)** (155, 180). The specificity of the TST is about 99% in non-BCG-vaccinated

populations with little exposure to NTM (144) but decreases to 95% where cross-reactivity with NTM is common **(I)** (155).

Positive TST reactions are common after completed treatment of active and latent TB (164, 205). The possibility of false positive TST reactions after eradication of infection without treatment has been suggested (206), although the extent of this phenomenon is unknown. Consequently, estimates of the prevalence of LTBI may be exaggerated even when the influence of BCG vaccination and NTM infections has been accounted for.

Comparative skin testing

Comparative skin testing with sensitin and tuberculin has been used to evaluate the influence of NTM infections on TST reactivity. In this method, each antigen is injected simultaneously by the Mantoux technique on either forearm, and reactions after 48-72 hours are compared. The antigen that causes an induration larger than the other is denoted dominant and indicates the etiology of the infection.

Epidemiological studies in the United States in the 1950's showed that individuals who reacted with small reactions (ranging from 3-11 mm) to PPD-S had mostly sensitin-dominant or equal reactions (207). In contrast, individuals with PPD-S reactions of ≥12 mm or more were mostly tuberculin-dominant. The frequency of large reactions varied with other evidence of tuberculous infection, while the frequency of smaller reactions varied primarily with geography, suggesting non-tuberculous etiology. A following large survey of US navy recruits confirmed the association of tuberculin-dominant reactions with tuberculous infection: in individuals with TST indurations in the range of 6-11 mm, tuberculin-dominant reactions were associated with a nearly 10-fold higher risk of TB than reactions that were sensitin-dominant (208).

Varying criteria have been used to define a dominant reaction, based on size of the dominant reaction as well as on size difference (138, 139, 209). In addition, the sensitins and tuberculins used differ between studies. Sensitins produced from *M. avium* are the most widely used, since *M. avium* is generally the most widespread cause of NTM disease. Most patients with pulmonary disease caused by *M. avium* had *M. avium*-dominant reactions (138). This finding supports the use of *M. av*ium-dominant reactions also in healthy individuals to indicate infection due to *M. avium* (199). Many NTM are antigenically closer to *M. avium* than to *M. tuberculosis*, and cross-reactions with *M. avium* sensitin are therefore more common than with tuberculin. Consequently, *M. avium*-dominant reactions can be extended to indicate other NTM infections as well, rather than tuberculous infection (208).

A recent review by Farhat (198) concluded that NTM infections have little influence on TST reactivity in high- and medium-prevalence populations, but may be an important cause of false positive TSTs in low-prevalence areas where NTM infections are common. Thus, results from Sweden **(I)**, the Netherlands (137) and southern parts of the United States (199) indicate that about 50% of TST reactions in adults of 10-14 mm are related to NTM infections. Significant influence of NTM infections on TST reactivity is also seen in Swedish children (93). According to the review by Farhat, on average only 2% of NTM-infected individuals in low- as well as high-prevalence countries have TST reactions ≥10mm.

THE DEFINITION OF A POSITIVE TUBERCULIN SKIN TEST

The main purpose of the TST is to detect tuberculous infection. For individuals with a normal immune system, test sensitivity is high (178, 184, 187, 200), whereas the specificity varies depending on the rate of false positive tests induced by BCG vaccination or NTM infection. If such false positive reactions are common in low-prevalence settings (where true positive reactions due to tuberculous infection is rare) most positive TSTs will be false, and the positive predictive value will consequently be low.

The sensitivity and specificity of the TST are also dependent on the cut-off value used to define a positive test. A higher cut-off value would result in fewer false positive reactions and an increased positive predictive value, although at the expense of decreasing test sensitivity. In contrast, if sensitivity is given priority, a lower cut-off value may be chosen, resulting in fewer false negative reactions. Sensitivity should be a priority in individuals with a high likelihood of tuberculous infection, such as close contacts to smear-positive patients, but also in individuals with increased risk of developing TB once infection is established. Examples of the latter are the immunocompromised and individuals recently exposed to TB. Such reasoning is the basis for the use of three different cut-off values, as is recommended in the United States for the 5 TU PPD products (144). Reactions of ≥5 mm are considered positive for those at highest risk, ≥10 mm for those at intermediate risk, and ≥15 mm for those at low risk.

The cut-off value for a positive reaction for PPD RT23 is 6 mm as recommended by the manufacturer (210). This recommendation is based on the frequency distribution of TST reactions in TB patients and non-vaccinated individuals with low risk of NTM infection, as observed in the above-mentioned epidemiologic studies from the 1940s and 1950s (178). The frequency distribution in such populations has its anti-mode at 5-6 mm**,** which constitutes a natural dividing-line between the infected and the non-infected

population. Consequently, sensitivity was the priority when the cut-off value of 6 mm was recommended. However, in populations with high rates of BCG-vaccination or NTM infection, a cut-off of 6 or even 10 mm may result in a large proportion of false positive reactions, i.e. the specificity may be low **(I)** (203, 204, 211). If the prevalence of tuberculous infection in such populations is low, the utility of the TST will be limited due to a low positive predictive value. In contrast, the positive predictive value using the 6 mm cut-off (or 5-10 mm for 5TU PPD products) may still be high, in spite of a relatively low specificity, in e.g. individuals from high-prevalence areas or close contacts of smear-positive cases (187, 203, 212-214). Consequently, the official statement of the WHO regarding the TST (215) leaves no recommendation of a specified cut-off value for a positive test. Rather, it is stated that decisions of the cut-off value should be based on the distributions of reactions in TB patients and the general population, as well as on the purpose of the test.

Considering the profound changes in the epidemiology of mycobacterial infections in many countries during the last decades, updated information on TST reactivity in the population is needed for evidence-based recommendations on the interpretation of the TST. Specifically, reconsideration of cut-off values requires quantification of the current sensitivity and specificity in the population (155). A study of the sensitivity of the TST is suitably conducted in TB patients or close contacts of patients with contagious TB. The specificity can be estimated in subjects with a very low risk of exposure to TB, in which nearly all TST reactions are non-specific (**I**) (155). With the sensitivity and specificity defined, predictive values of positive and negative test results can be estimated for different assumed prevalences of tuberculous infection (155). Based on such estimates, appropriate cut-off values for a positive test can be chosen depending on the population tested and the purpose of the test.

GENERAL EPIDEMIOLOGICAL FACTORS ASSOCIATED WITH TUBERCULIN SKIN TEST REACTIVITY

The interpretation of the TST is complex, and knowledge of the influence of background factors facilitates the process. In addition to natural exposure to mycobacteria and previous BCG vaccination, other factors may be of importance, such as age, gender, country of birth, smoking habits and socioeconomic factors. The relative influence of these parameters varies between populations and is valuable to know for the clinician when assessing a TST reaction.

Age

The abiltiy to mount a DTH response is not fully developed in newborns (216, 217). After infancy, this ability does not vary with age until after about 65 years, when false positive TSTs due to anergy become increasingly common (141, 174, 213). However, TST reactivity increases with age, as the probability of mycobacterial exposure increases **(I, II)** (38, 204, 218-220). The prevalence of tuberculous infection increased markedly with age in European children at the start of the 20th century, and by the age of 20 almost everybody was infected (38). The age-related increase of tuberculous infection in high-endemic countries today is not as steep, with prevalence rates of 50% in 30-year olds in e.g. sub-Saharan Africa (38). After the rapid decline in TB rates in industrialized countries during the last century, TST reactivity in European children today is very low, as well as the age-related increase. These reactions are predominately caused by NTM infections, which also become more common with age (221-223). Recent findings suggest that the age-related prevalence of NTM infections continues to increase in adults (88) and contributes to the age trend in TST reactivity **(I)**.

Gender

TST surveys in different settings during the pre-BCG era consistently showed that the prevalence of LTBI is higher among males than females after about 15 years of age (224). This gender difference may be a result of different social mixing patterns. An alternative explanation for these findings is that there are biological gender differences in DTH to mycobacterial antigens (225). Dolin reviewed the frequency distributions of TST surveys of non-BCG-vaccinated high-endemic populations (226) and found modes and antimodes for males and females at corresponding induration sizes. He therefore argued against a biological difference in DTH reactivity, but proposed that hormonal factors may protect post-adolescent females from infection.

No gender differences were revealed in neither non-vaccinated nor BCG-vaccinated children and adults in Sweden (**I**, **II**) (86). However, several studies of low-endemic populations with high rates of BCG vaccination have found larger TST reactions in males than females (204, 218, 219). The latter findings add support to the theory of a biological gender difference in DTH. Gender differences have also been shown in the development of active disease. Females in their reproductive years have a higher progression rate from infection to disease, whereas men have higher rates of progression at older ages (224).

Country of birth

TST reactivity in immigrants reflects the TB incidence in their country of origin **(I)** (40, 41, 227-229).

Socio-economic factors

TB as a disease of poverty is a well-established concept (230). The correlation between prevalence of tuberculous infection and socio-economic factors was observed early in the 20th century (231). Later studies have pointed out that positive TST reactions are related to the socio-economic status of neighborhoods (232) as well as to crowded housing and the education level of parents (233).

Smoking

According to two recent reviews, smoking is a risk factor for tuberculous infection, as shown by a positive TST, as well as for active TB (234, 235). In addition, evidence suggest that passive exposure to tobacco smoke in children is associated with an increased risk of tuberculous infection (236) and pulmonary TB (237).

INTERPRETATION OF REPEATED TUBERCULIN SKIN TESTS

In addition to periodic TSTs in surveillance of individuals at risk, the TST is often repeated in contact tracing when an exposed person is TST negative at the first examination. The purpose of this procedure is to detect newly developed DTH to tuberculin, "TST conversion", as a sign of recently acquired tuberculous infection. Theoretical aspects of the interpretation of repeated TSTs are discussed below.

Biologic variation and differences in administration and reading of the TST will result in a standard deviation of less than 3 mm (238). Consequently, when repeated tuberculin tests are given, random variation should result in differences of less than 6 mm (representing 2 standard deviations) in 95% of subjects. A criterion of 6 mm is therefore appropriate to distinguish increases in reaction size due to random variation alone from true biologic phenomena (141).

Although skin testing with tuberculin does not induce DTH to tuberculin on subsequent tests, waned hypersensitivity from remote mycobacterial infections can be boosted. Thus, the stimulus of a first test may increase the size of the reaction to a second test in subjects previously infected with mycobacteria

(tubercle bacilli, NTM or BCG) (239). According to one hypothesis, the booster phenomenon occurs when the number of memory T cells is too low for a full response to the initial injection of tuberculin. When the memory T cells proliferate during the initial response, more of them are available on the second test, which evokes a larger reaction (141). Boosting is maximal 1 – 5 weeks after the first test (240) and can be still be detected after one year (141, 241-243). In one study a booster effect was detected up to five years after the first test (244).

The booster phenomenon is sometimes defined as a negative TST reaction which becomes positive on subsequent testing in the absence of new mycobacterial infection (245), whereas most authors add the criterion of an increase by at least 6 mm to allow for the inherent variability of the test (241, 242, 246-251). Unlike positive reactions to an initial TST, boosting is generally not associated with previous TB exposure (240, 247), since TST reactivity due to latent TB is relatively persistent (175). However, a strong correlation has been observed with a history of BCG vaccination (240, 243, 246-248, 252) and reactivity to antigens of NTM (240, 242, 247, 253). The prevalence of the booster phenomenon ranged from 6-31% in those of the mentioned studies which used the criterion of a 6 mm increase from a negative to a positive reaction (240, 246-248, 253). Boosting has been observed in BCG-vaccinated children (246, 248) but becomes more common with advancing age (241, 242, 247).

The term “conversion” refers to the development of DTH to mycobacterial antigens following BCG vaccination or infection with tubercle bacilli or NTM in a previously non-sensitized person. A commonly used operational definition for *M. tuberculosis*-induced conversion is an increase of at least 10 mm within a period of two years (144). Conversion in the context of TB exposure has important clinical implications, as it is associated with a high rate of TB the following two years (38, 164, 172, 254, 255). The period between the last exposure and the second TST should be a minimum of eight weeks in order to detect all conversions (141).

When initiating periodic skin testing, a two-step TST has been recommended in order to avoid future false TST conversions due to the booster phenomenon (144). In this method, individuals with a negative initial TST undergo a second test 1–4 weeks after the first test. The result of the second test is then taken as the baseline with which to compare future TST reactions (241, 249-251, 256, 257).

FUTURE RISK OF TUBERCULOSIS IN NON-BCG-VACCINATED SUBJECTS RELATED TO TUBERCULIN SKIN TEST REACTIVITY

A large number of studies from a wide range of different settings have established a positive association between TST reactivity and subsequent risk of active TB (reviewed in (141, 167, 258)). Most of these studies were carried out in non-BCG-vaccinated populations, or the results were controlled for the effect of BCG. The lifetime risk associated with a tuberculous infection, as determined by a positive TST, is often approximated to 10% (38, 144, 259). However, many factors modulate the risk of progression to disease in an infected individual, such as time since infection, age, size of the TST reaction and medical conditions. Consequently, a lifetime risk of 10% may be a substantial underestimate for many individuals (260). Furthermore, if a definition of a positive TST with low specificity is applied in studies of TB risk, non-infected subjects are included in the population defined as infected, and the incidence of TB will be underestimated. Such an effect can be seen in studies based on TST conversion, in which many subjects with false positive boosted reactions may be included (6).

The time factor

Individuals with recent infection, as defined by TST conversion, are at high risk for progression to disease shortly after infection has occurred. Fifty to eighty percent of the estimated lifetime risk occurs during the first 1-2 years after infection, after which the risk rapidly decreases (164, 167, 172, 254, 255, 259, 261). Without intervention, 2 - 5% of contacts with newly acquired tuberculous infection develop TB within 2 years of the exposure.

The age factor

Studies performed in the early twentieth century showed that infants with tuberculous infection had a 40% risk of developing disease - often serious, life-threatening forms - within 1-2 years (262). A high risk of TB related to a positive TST is also seen in children 1 – 4 years of age, at least in part due to the fact that their infections are recent (263). However, adolescents and young adults with a positive TST appear to be especially prone to disease progression (259, 263), whereas the ages 5-14 are relatively spared (262). In addition, Stead found a remarkably high risk of disease progression after TST conversion in the elderly, in which a positive TST without observed conversion also was associated with a high risk of TB (172).

The size factor

Since large TST reactions are more likely to be caused by tuberculous infection than smaller reactions, there is a clear positive correlation between the size of a positive TST reaction and the risk of developing TB (258, 264, 265). The correlation may also be caused by a more active cell-mediated immune response in infections that will lead to disease (266), possibly due to a larger infecting dose (174, 175). Alternatively, the correlation is due to larger reactions among those with more recent infection (267), consistent with the finding that the influence of size on the risk of developing TB is particularly large in children and adolescents and decreases with age in adults (260).

The health factor

Multiple clinical conditions are associated with increased risk for TB in TST positive individuals (159). HIV infection is the strongest known risk factor. Some risk factors are especially important due to their high prevalence, such as diabetes mellitus and apical fibronodular changes on chest radiograph consistent with prior TB. Other conditions with increased risk of disease progression are chronic renal failure, silicosis, intravenous drug abuse, being under-weight, certain malignancies and immunosuppressive treatment. Among immuno-suppressive drugs, TNF-α-blocking agents are associated with a particularly high risk (71).

PROTECTIVE IMMUNITY AGAINST TUBERCULOSIS RELATED TO TUBERCULIN SKIN TEST REACTIVITY

Protective immunity against reinfection with tubercle bacilli

Animal studies have consistently shown that tuberculous infection confers effective immunity against developing TB from reinfection (268). Corresponding protection in humans with tuberculous infection, as indicated by a positive TST, has been observed following intense exposure to infectious cases (6). The protection is likely mediated by prompt activation of macrophages by memory T cells available after the previous infection (269).

Among the first to systematically study this issue were Olaf Scheel and Johannes Heimbeck at Ullevaal Hospital in Oslo. They administered a mandatory TST program (using the Pirquet method) for student nurses at entry during the period 1924-1936, in which TB was highly prevalent in Norway. About half of the students were TST negative at the time of entry, but due to heavy exposure to TB at the hospital, nearly all of them became infected and

converted their Pirquet test during the 3 years of training (270). One third of the initially Pirquet negative students developed TB, half of them during their first year of training, whereas TB occurred in only 3% of the Pirquet positive students (after excluding those with a history of TB or evidence of TB on entry) (8). Likewise, analyses of hospital outbreaks of TB show a strikingly low risk of TB after exposure in TST positive individuals, before (271) as well as after (6) the introduction of preventive therapy for LTBI.

It can be argued that those who have escaped infection without developing disease constitute a selected group with TB resistance better than average. However, as in the studies by Scheel and Heimbeck, TST negative individuals who were BCG-vaccinated had a similarly low risk of TB during follow-up as those with positive TSTs and thus achieved equal protection (8). It may therefore be concluded that the immune response to a tuberculous infection does indeed confer increased protective immunity against reinfection in a healthy person (6, 38, 144). This protective effect has been estimated to 80% from studies of healthcare students and workers (272) as well as from epidemiogical data (273), corresponding to the maximum protective effect of BCG vaccination (274) (see p. 55).

Protective immunity from infection with non-tuberculous mycobacteria

The potential of an immunizing effect of NTM against TB stems from the presence of shared antigens in NTM and tubercle bacilli (195), which are capable of eliciting cross-reactions with memory T cells primed by antigens from NTM. This phenomenon is termed heterolgous immunity (275) (as opposed to homologous immunity when the immune response is caused by reactivity to antigens from the same species).

Several lines of evidence indicate that protective immune responses against TB can be evoked by heterologous immunity to NTM infections (275). Experiments in guinea pigs demonstrated that infections with *M. avium* and other NTM increased the animals' resistance against subsequent challenge with *M. tuberculosis* in varying degrees (276, 277). Evidence in humans was found e.g. in large epidemiological studies in which individuals with small or intermediate-size TST reactions, indicative of NTM infection, were at lower risk of TB than those with no TST reactivity (202, 258, 266, 278). In the previously mentioned study of US navy recruits, in which comparative skin-testing was performed, the lowest TB risks were found in individuals with TST reactions of 6-11 mm whose reactions were sensitin-dominant (208). Corresponding protection was found in British adolescents who reacted only to the 100 TU dose of tuberculin (278) - reactions which are also indicative of NTM infection (184). Finally, a study by Ravn (279), using in vitro correlates

of protection (see p. 67), also supports the induction of a human protective immune response by NTM.

PROS AND CONS OF THE TUBERCULIN SKIN TEST

Pros

- Non-technological performance - does not require a laboratory
- Low material costs
- Well standardized
- High sensitivity in healthy individuals
- High specificity in non-BCG-vaccinated populations
- High efficacy in populations with high prevalence of TB
- Well-documented prognostic significance
- Documented selective efficacy for treatment of LTBI

Cons

- Low sensitivity in the immunocompromised
- Low sensitivity at diagnosis of active TB
- Low specificity in BCG-vaccinated populations
- Cross-reacts with NTM infection
- Age-dependent results
- Elicits a booster phenomenon at repeated tests
- Instability over time (reversion in spite of LTBI)
- Requires a second visit after 48-72 hours
- Requires skilled testers
- Reading susceptible to bias and digit preference
- Reagent sensitive to heat and light

NEW IMMUNOLOGICAL DIAGNOSTIC TESTS FOR TUBERCULOUS INFECTION

The TST was for nearly a century the only test available for the diagnosis of tuberculous infection. Recently, advances in immunobiology have led to the development of new diagnostic tools, the IFN-γ release assays (IGRAs). The basic principles for these tests and their performance are briefly reviewed below.

During the attenuating process of the *M. bovis* strain that produced the new strain BCG, several genetic segments were lost, one of which is designated Region of Difference 1 (RD1) (280). Two highly antigenic proteins are encoded in this region: the 6 kDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10). The ability of these antigens to stimulate IFN-γ production by T cells *in vitro* form the basis for the IGRAs. Two IGRAs are licensed for commercial distribution. T-SPOT.TB (Oxford Immunotec, Oxford, UK) uses an enzyme-linked immunospot assay (ELIspot) to detect IFN-γ-producing T cells after separation of peripheral blood mononuclear cells. The other test, QuantiFERON-TB Gold In-Tube (QFT) (Cellestis, Victoria, Australia), uses an enzyme-linked immunosorbent assay (ELISA) to measure the production of IFN-γ by circulating T cells in whole blood. This latest version of QFT includes a third RD1 antigen, TB7.7 (281).

The sensitivity of IGRAs in newly diagnosed active TB appears to be comparable to or slightly better than that of the TST (180). The sensitivity and specificity in diagnosing LTBI is difficult to determine in the lack of a gold standard. The fact that RD1 antigens are not shared with BCG substrains avoids false positive results in BCG-vaccinated subjects. IGRAs consequently have a higher specificity than the TST in populations with high rates of BCG vaccination (180, 282). The diagnostic antigens are generally absent in NTM (although ESAT-6 and CFP-10 are included in *M. kansasii*, *M. szulgai* and *M. marinum)*, potentially causing lower rates of false positive results in areas where NTM exposure is common (201, 283). Other important advantages compared to the TST are avoidance of reading bias and booster reactions as well as the need for only a single visit.

Obvious limitations of IGRA tests are the high material costs and the need for laboratory resources, and several uncertainties remain regarding test performance. Further evaluation is needed in children, the elderly, and in individuals with immunosuppressive conditions (180). A major disadvantage compared to the TST is the uncertain prognostic value for progression from tuberculous infection to active TB (284-286), and treatment efficacy of latent

TB based on IGRA results waits to be demonstrated. Furthermore, there is uncertainty regarding the stability of IGRA results in serial testing (287) as well as regarding the performance in subjects with remote tuberculous infection. Nevertheless, the Center for Disease Control and Prevention (CDC) in the United States has recommended that QFT can be used in any situation where the TST is used, including serial testing of HCW (288). In Europe the guidelines from National Institute for Clinical Excellence (NICE) in the United Kingdom recommend that IGRA tests are used to confirm a positive TST (117). However, it should be remembered that in spite of the high specificity of IGRA tests, the positive predictive value will still be poor in low-prevalence settings. Consequently, IGRAs are not suitable for the purpose of non-targeted screening.

BCG VACCINATION

BCG STRAINS AND VACCINE PRODUCTION

The BCG strains in current use are all descendants of the original BCG strain produced by Calmette and Guérin in 1921. The original strain was sent to many laboratories around the world in which it was propagated under varying culture conditions. This went on for about 40 years and resulted in a variety of new BCG strains, until further changes could finally be prevented in the 1960's by a combination of seed-lot and freeze-drying methods (289). The substrains had by then suffered spontaneous mutations in such a way that there were clear differences in the macroscopic properties of strains from the different laboratories (169). Eight mutations have been identified and are present at various degrees in the substrains in use today (290). The substrains differ in immunogenicity in animal models (291), but whether any substrain is superior to others in the protection of humans has not been determined (10). However, a difference has been observed between substrains and the incidence of adverse reactions (169). Other differences between substrains are reactogenicity (measured in terms of the size of the local lesion) and the ability to induce DTH (169). These qualities are also affected by considerable differences in the numbers of viable and dead organisms between different BCG products (169).

VACCINATION TECHNIQUES

BCG was first administered orally, but this route required large doses (292). Attempts with the subcutaneous route resulted in a high frequency of large abscesses. Arvid Wallgren in Göteborg therefore introduced the intradermal route (293), which produced a lower frequency of more superficial abscesses and eventually became the most common administration method. The recommended injection site is the deltoid insertion region of the upper arm (168). A raised pale bleb is the sign of correct injection. A 25-27 gauge needle and a low-volume syringe are recommended, capable of accurately delivering 0.05 ml to infants and 0.1 ml to older children and adults.

In addition to the intradermal method, percutaneous administration with multiple puncture devices is used in some countries. The relative efficacy of percutaneous vs. intradermal administration is unknown. Comparisons with TST reactivity and in vitro correlates of protective immunity (see p. 66) have shown divergent results (294-296).

BCG POLICIES AND COVERAGE

Global policy and coverage

Mass BCG vaccination was introduced in Europe during the 1940s, targeting newborns and school children who were TST negative. Large vaccination campaigns of children were subsequently spread around the world, followed by routine childhood vaccination in most countries. The United States (116), the Netherlands (297) and Iceland (298) never adopted a general BCG vaccination policy, due to skepticism about its efficacy and concern about interference with the TST.

At present the WHO recommends neonatal BCG vaccination in countries with a high prevalence of TB (168). BCG vaccination is also recommended for children at increased risk of TB exposure in low-endemic countries and for those exposed to MDR-TB. Consequently, BCG is one of the most widely used vaccines in the world. Worldwide coverage is estimated at over 100 million doses per year, resulting in vaccination of 76% of all children born in 2002 (299).

Most countries follow the WHO recommendation to give only a single dose of BCG at birth or at earliest contact with a health service. However, many countries have developed their own policies, such as giving BCG to older children, targeting only high-risk groups, giving repeated vaccinations, or not using BCG systematically at all (11, 299, 300).

Swedish policy and coverage

In Sweden the numbers of newborns who were BCG-vaccinated increased during the 1940's and reached 95% in the 1950's and onwards (301, 302). In addition, primary and repeated vaccinations were offered to TST negative 7- and 15-year old school children as well as to military recruits. While these preventive measures were intensified, there was an ongoing rapid decline of the incidence of TB, and as early as 1955 Arvid Wallgren raised the question if the general BCG vaccination should be discontinued (303). Vaccination of 7-year old school children ended in 1965, but it was not until April 1975 that the general vaccination of newborns finally was replaced by selective vaccination of groups at increased risk of TB (12). Crucial to this decision was, in addition to the declining risk of infection, an increased frequency of BCG-induced osteomyelitis that occurred following the transfer of production of the Swedish BCG strain (304) from Göteborg to Copenhagen (305, 306). The production of this BCG vaccine was discontinued in 1979, and since then BCG Danish1331, produced at Statens Serum Institut, has been used. Due to a continued decline in

the risk of tuberculous infection and disease, vaccination of army recruits was ended in 1979 (302) and vaccination of 15-year old school children was ended in 1986 (307).

Vaccination coverage of newborns fell to less than 2% following 1975, but after intensified information to healthcare providers the coverage gradually increased from 1982 (12). Levels around 15% were reached in the 1990's and in 2007 the rate had increased to 18% (30), reflecting the increased proportion of immigrant families in Sweden. Romanus estimated that 88% of newborns belonging to the targeted risk groups were vaccinated during the period 1998-2002 (12). The recommended age of vaccination was changed in 1994 to 6 months or older, in order to avoid vaccination of infants with severe immunodeficiencies (308). However, neonatal vaccination is still recommended for children with particularly high risk of TB exposure, provided no signs of immunodeficiency are revealed among close relatives. Newborn children with known exposure to pulmonary TB are treated with e.g. isoniazid for 2-3 months, and a decision regarding BCG vaccination is delayed until at least 3 months of age. Children with a negative TST are then BCG-vaccinated, whereas those with a positive reaction complete the full course of treatment.

All students and employees in healthcare settings were until recently included in the Swedish definition of groups at increased risk of TB exposure (309). However, in the recommendations from the National Board of Health and Welfare (Socialstyrelsen) from 2006 (118), BCG vaccination is encouraged only for workers and students at clinics of respiratory medicine or infectious diseases, TB laboratories and pathology departments.

IMMUNOLOGICAL RESPONSE

Systemic protective reaction

In spite of its long history and extensive use, the protective mechanism of BCG is poorly understood. A few points from research in vaccine immunology will be mentioned.

BCG vaccination normally leads to an asymptomatic bacteremia. Animal studies and autopsy studies of BCG-vaccinated children who died of other causes than TB indicate that BCG and granulomas are distributed widely in many organs (310). The fact that disseminated BCG infection can occur years after vaccination in HIV patients (311) suggest that viable organisms may persist for long periods.

Within 8 weeks of vaccination a cellular immune response to mycobacterial antigens can normally be detected *in vivo* by the TST, as well as by *in vitro* methods. Studies in neonates (295, 312-314), adolescents (315) and adults (**IV**) (279, 316) show that BCG vaccination induces a potent Th1-type memory immune response, characterized by mycobacterium-induced IFN-γ production and lymphocyte proliferation, as well as a specific cytotoxic T cell response (312). Thus, the immunological events of a tuberculous infection in a BCG-vaccinated individual are similar to the events of a reinfection in a non-vaccinated individual (317).

Animal experiments suggest that vaccination with BCG protects against uncontrolled replication and dissemination of tubercle bacilli but not against acquisition of infection (318, 319). In humans, neonatal BCG vaccination is highly protective against disseminated and meningeal disease (20), but less protective against other forms of disease (274), implying a similar protective effect as in mice. Whether BCG protects against acquisition of infection has been difficult to investigate in humans, since studies using the TST cannot, in the individual case, reliably differentiate between tuberculous infection and BCG vaccination (214, 320). However, IGRAs have the potential to make this differentiation (see p. 42). In a prospective study of children with recent household TB exposure, Soysal and colleagues estimated the protective effect of BCG vaccination against infection, using ELISpot results as a marker of infection (321). They found that presence of a BCG scar was independently associated with a 24% reduction in risk of infection, suggesting that some of the protection provided by BCG vaccination may be attributable to prevention of infection. In contrast, an autopsy study of BCG-vaccinated and non-vaccinated subjects who had died from other causes than TB suggested that BCG does not prevent infection in humans; no decrease in the likelihood of primary lesions in the lungs was observed among the BCG-vaccinated subjects in this study (322).

Post-vaccinal lesion

The local response to an intradermal injection of BCG typically develops along a common course of events. The extent of the reaction is variable and may be influenced by the age and immune status of the vaccinee, the skills of the vaccinator, the BCG strain and dose of the vaccine (323).

Vaccination of newborns and TST negative children regularly results in a local reaction with erythema and tenderness. In the second week, a small induration develops, followed by a softening process of the central area, which gradually turns into a yellow pustule during the second month and finally leads to the formation of a crust. When this crust falls off, an ulcer with a diameter of <10 mm appears that slowly heals during the third month (316, 324, 325). In a few

cases (<5%) a transient, moderate adenitis may be seen in the regional lymph nodes (21), particularly in the axillary nodes. The size of the lesion has been used as a quality indicator of BCG products (169, 326) and of vaccination procedures in the field (170), in addition to the TST.

BCG vaccination of subjects with tuberculous infection elicits a more rapid reaction called the Koch phenomenon (327). In this type of reaction, an induration develops within 48 hours, an ulcer within a week, and a crust within the next week. There may be more discomfort compared to TST negative subjects, but no increase in lymph node reactions or other adverse effects have been observed. The resulting scar is generally larger and there is a higher frequency of keloid formation (327-329). Repeated BCG vaccination in school children in Brazil caused an intense and early reaction similar to the Koch phenomenon, but the time of healing was not reduced compared to a first dose (324).

BCG vaccination without a preceding TST was recommended by the WHO in 1964 (330), with the intention to thereby increase vaccination coverage. However, in Sweden and other developed countries, increased scarring and intensity of the post-vaccinal reaction in TST positive subjects motivates a pre-vaccination TST in older children and adults.

Due to its ability to induce a Koch phenomenon, a BCG injection may be used to diagnose tuberculous infection. Although useful in children according to several studies (reviewed in (327)), an investigation performed by the WHO did not corroborate these findings (329). Furthermore, BCG vaccines are not standardized for diagnostic purposes; the extent of the lesion varies significantly between strains (329). Consequently, BCG as a diagnostic reagent is not officially recommended (329, 331).

BCG scar

The healing process of the post-vaccinal lesion generally results in a characteristic scar. The typical BCG scar is circular, superficial and slightly depressed, with atrophic, smooth skin and irregular edges. Scar formation is related to the extent of the post-vaccinal lesion (332, 333), and hence correlated to the same background factors, i.e. age and immune status at vaccination, the vaccination technique and the strain and dose of the vaccine (323). The scar one year after vaccination of Swedish adults was somewhat smaller than the lesion as measured after 2-3 months **(II)**, whereas the opposite trend was observed in children in other studies (217, 332, 334).

A relatively low prevalence of scars was seen in BCG-vaccinated children in various countries (217, 225, 323, 335), whereas a scar was identified in more than 90% of children in other studies (336-339). The scar rates in Swedish children are among the lowest reported in the literature. Scars were observed in 59% of children vaccinated as neonates in 1971 (340) and in 51% of children vaccinated at varying ages in the late 1980's **(II)**. Several studies indicate less scar formation after vaccination in the first month of life compared to later vaccinations (217, 225, 323, 341, 342), possibly due to a relative immaturity of the neonatal immune system (24, 217) and/or the low dose of BCG generally used in newborns (24). However, neonatal vaccination does not fully explain the differing scar rates between studies.

The propensity of scar formation increases with age at vaccination (225). The prevalence of scars is generally >90% after vaccination of older children and adults (225, 334, 343), and consistent scar formation was seen after BCG vaccination of Swedish young adults **(II)**. A likely reason for increasing scar formation with age is an age-related increase in mycobacterial exposure prior to vaccination, resulting in a memory response that amplifies the vaccine reaction and the following scarring process. Thus, scars were larger after vaccination of TST positive children than after vaccination of children who were TST negative (328, 329). Likewise, BCG lesions (333) and scars (225) were larger after revaccination (i.e. after previous exposure to BCG) than after primary vaccination, and the size of the lesions correlated with the TST reactivity before vaccination in one study (333).

BCG scars can usually be distinguished from vaccinia scars as well as scars from bites and other injuries. Although some scars may be doubtful regarding their etiology, and a significant proportion of false positive scar readings was observed in one study (323), a typical scar is a useful indication of past BCG vaccination (24, 338). However, *absence* of a scar does not exclude past vaccination. Consequently, case-control studies based on BCG scars as proof of previous vaccination may underestimate vaccination efficacy, since the group defined as non-vaccinated may include a significant proportion of vaccinated subjects **(II)** (225).

Tuberculin skin test reactivity

As previously mentioned (on p. 31), tuberculin skin testing of BCG-vaccinated individuals can induce skin indurations caused by cross-reactivity between antigens present in tuberculin and BCG. False positive reactions due to previous BCG vaccination may cause considerable problems in the interpretation of TST results, in particular in low-endemic settings with high rates of BCG vaccination (204, 211). A typical situation in which this problem occurs is when the TST is performed in new HCW. Although general BCG vaccination was discontinued in Sweden over 30 years ago, many young people today have been BCG-vaccinated, e.g. immigrants and second generation immigrants. Knowledge regarding the influence of previous BCG vaccination on TST reactivity is therefore important.

The effect of BCG vaccination on TST reactivity varies considerably between different settings and may depend on several factors:

Genetic disposition and nutritional status

A small percentage of individuals will not respond to tuberculin following primary or multiple vaccinations. A genetic regulation of DTH to tuberculin is suggested by studies of human leukocyte antigen (HLA) class II phenotypes (344) and comparisons of TST reactivity in twins, siblings and unrelated children (345, 346). A poor response to tuberculin may also be caused by malnutrition, which has an inhibitory effect on DTH (347, 348).

BCG substrain

BCG substrains have varying ability to induce TST reactivity (10). Some potent strains can produce distributions of TST reactions similar to the distributions seen after tuberculous infection (332, 349), whereas others show lower conversion rates (10, 332). The Swedish BCG strain, used in Sweden until 1979, had a conversion rate of >90% (340). The conversion rate of the strain used in Sweden since then, BCG Danish 1331, is less (217, 350), although not well known.

Other vaccine-related factors

Storage of a BCG vaccine at 30°C reduced most of its sensitizing potency, whereas storage at room temperature had a minor weakening effect (171). One hour of sun exposure reduced the viability of bacilli by a factor of x1000 and TST reactivity was reduced by half (171). A decrease in the

vaccine dose reduced TST reactivity, but the effect was modest (171, 351); decreasing the dose by half only reduced reactivity by 1-2 mm.

Exposure to non-tuberculous mycobacteria prior to vaccination

Guld showed that prior infections with NTM resulted in larger TST reactions after BCG vaccination (352). However, Andersen recently suggested that exposure to NTM prior to vaccination may accelerate the waning of TST reactivity in tropical areas (353) (see p. 58)).

Previous BCG vaccination

Nyboe found that TST reactivity two months after vaccination was stronger in revaccinated than in primary-vaccinated children (333). Furthermore, TST reactivity was clearly correlated with the number of BCG scars in two studies (243, 354), although the number of scars in these studies possibly was confounded with age when vaccinated or time since vaccination. The effect of revaccination on TST reactivity could not be separated from the effect of age at vaccination in school children and young adults in Canada (355). Revaccination is considered an important reason for large TST reactions in individuals from countries of the former Soviet Union and Eastern Europe (356), where repeated vaccinations are common (300).

Age at vaccination and time since vaccination

TST reactivity after vaccination in infancy wanes rapidly according to many studies (141). Vaccination after the first year of life generally results in more persistent reactions (141). The relation between the influence of age at vaccination and time elapsed since vaccination is illustrated by Farhat's review of a large number of studies in which BCG-vaccinated subjects and controls were tuberculin skin-tested (198). In subjects who were BCG-vaccinated in infancy, TST reactions ≥10 mm attributable to BCG occurred in 6% of subjects overall and in only 1% of those who were tested after more than 10 years. According to the same review, subjects vaccinated after the first year of life had TST reactions ≥10 mm attributable to BCG in 42% of all subjects and in 21% of subjects tested after 10 years or more. Only few of the reviewed studies included adults in low-endemic countries, in which considerable TST reactivity related to neonatal BCG vaccination has been observed (see below)**.**

In Swedish children with a BCG scar, positive TST reactions (≥6 mm using PPD RT23) were seen in 74% of preschool children and in 55% of children

aged 8-9 years **(II)**. About half of the children had been vaccinated in the year of birth, suggesting a relatively slow waning process.

Mycobacterial exposure after vaccination

Waning of vaccine-induced DTH may be counteracted by subsequent mycobacterial exposure. Such influence on TST results is suggested by a study of children in Saudi Arabia (357). While TST reactivity was equal in BCG-vaccinated and non-vaccinated children at the age of 5 years, the increase of reactivity with age was more pronounced in the vaccinated children, so that the difference between the two groups became statistically significant in those aged 12 and 13 years. In low-endemic areas, a stimulating effect of mycobacterial exposure on BCG-induced TST reactivity is likely to be caused by NTM rather than by tubercle bacilli, as indicated by studies of Swedish children (358) and young adults **(I)**. In the latter study, 62% of the BCG-vaccinated adults were TST positive (≥6 mm using PPD RT 23) and 42% had TST reactions of ≥10 mm. Corresponding figures of the non-vaccinated subjects were 5% and 3%, respectively. In the same study, vaccination in the year of birth resulted in considerable TST reactivity compared to non-vaccinated subjects. Other TST studies of adults in low-endemic countries have documented a substantial influence of neonatal BCG vaccination as well (218, 359). In conclusion, results from different settings indicate that mycobacterial exposure maintains or reinforces the effect of BCG on TST reactivity (203).

Repeated tuberculin skin tests

Repeated TSTs can increase and prolong BCG-induced TST reactivity due to the booster phenomenon (243, 246, 252, 360) (see p. 36).

Type of tuberculin

As previously mentioned (on p. 23), PPD RT23 was found to be more potent than PPD-S in eliciting reactivity in BCG-vaccinated subjects (129). A review of the effect of BCG vaccination on TST reactivity found a substantially stronger influence of BCG using PPD RT23 than for 5TU PPDs (361), while another review revealed no such difference (198).

BCG-induced TST reactions are generally smaller than those induced by tuberculous infection (361), and a TST reaction of ≥15 mm is recommended for the differentiation between reactivity due to BCG vaccination and tuberculous infection in low-risk populations (159). This recommendation is based on a large number of studies and is supported by a recent meta-analysis (361).

The strong influence of BCG vaccination on TST reactivity in low-endemic settings reduces the utility of the TST in screening of tuberculous infection. However, there are situations and settings in low-endemic areas in which the expected rate of tuberculous infection is high, as in certain groups of immigrants and in close contacts of smear positive TB patients. In such settings, TST reactivity correlates well with risk factors of TB exposure and the influence of BCG vaccination is limited (213, 228). The relative usefulness of the TST is thereby maintained in spite of BCG vaccination, corresponding to the situation in high-endemic areas (187, 212, 214, 267) (see p. 34). Consequently, it is prudent to disregard the influence of BCG vaccination in the interpretation of the TST in such settings (159). Following this principle will lead to a proportion of false positive reactions depending on what cut-off value is used. A follow-up IGRA test offers an obvious opportunity to reduce the number of false positive assessments (362), although more longitudinal studies are needed to establish the prognostic value of these tests.

Correlation between the BCG scar and tuberculin skin test reactivity

A correlation between the presence or size of a BCG scar and TST reactivity is usually observed in vaccinated populations, after recent (217, 363) as well as remote vaccination (**II**), (248, 332, 364-367). The strength of the correlation is variable, and in some studies no correlation is seen (335, 337, 368). Some of the variation may be vaccine-related: the extent of the local reaction is proportional to the total bacterial mass, while TST reactivity is related to the number of viable bacilli (169). With this exception, the background factors that determine scar formation also determine BCG-induced TST reactivity in a corresponding manner. Furthermore, a large survey of BCG scars in Malawi found detailed age-sex patterns of scar size that followed the pattern of TST reactivity (225). These observations suggest that DTH is involved in the process of scar formation, consistent with the close association between scar formation and TST reactivity.

ADVERSE EFFECTS

Experience from many decades of extensive use shows that BCG is a safe vaccine with a very low risk of severe complications.

The events associated with the post-vaccinal lesion are normal reactions to the inoculated infection and are generally considered as mild, in spite of often protracted discomfort and the generally high frequency of scarring. Local reactogenicity differs between strains (169).

The risk of suppurative lymphadenitis is increased in neonates (369) and is limited by the recommended dose reduction. Several outbreaks have been reported after shifting of BCG strain (169). A retrospective study by Romanus of Swedish children reported suppurative lymphadenitis in 0.9/1000 subjects (308). In the same study, vaccination site abscesses were noted in 0.4/1000 subjects. Subsequently, Romanus presented preliminary prospective data in her thesis (370) indicating substantially higher rates of the corresponding adverse reactions. One percent of the children in the latter study were referred for medical advice concerning the vaccine reaction.

BCG osteomyelitis is a generally rare complication which typically occurs within 7-24 months of vaccination (305). Outbreaks have occurred after shifting of BCG strain in Scandinavia (305, 306) and Czechoslovakia (369). In Sweden only a few cases of suspected osteomyelitis have been reported after 1979, none of them bacteriologically confirmed (12).

Disseminated BCG infection is a potentially fatal complication in immuno-compromised subjects. It was very rare before the era of acquired immunodeficiency syndrome (AIDS), when it generally was associated with other immunodeficiencies such as severe combined immunodeficiency (SCID), chronic granulomatous disease or IFN-γ receptor deficiency (371). Between 1979 and 1991, 4 cases of disseminated neonatal BCG infection occurred in Sweden among 100000 infants vaccinated at birth (308). Three of the infants were subsequently diagnosed with SCID. No further cases of fatal neonatal disseminated BCG infection have been reported after the changing of the recommended age of vaccination to 6 months (12).

A previously unrecognized high risk of disseminated BCG infection was recently estimated in HIV-infected infants in South Africa (372). The WHO therefore recommends that BCG should not be given to children who are known to be HIV-infected, but the vaccine is still recommended regardless of infant HIV exposure in settings with limited diagnostic resources (373). Finally, immune reconstitution infection syndrome (IRIS) is an important new

complication of BCG in HIV-infected children who are treated with anti-retroviral therapy (374, 375).

PRIMARY VACCINATION

Protective efficacy

The efficacy of BCG is consistently high against disseminated forms of TB in small children. Summary estimates of a BCG-induced protective effect against miliary or meningeal TB in randomized controlled trials was 86% (20) and in case-control studies 75% (20, 299). The 100 million BCG vaccinations given to children every year were estimated to prevent 30 000 cases of TB meningitis in infants during their first five years of life and an additional 11 000 cases of miliary TB (299). However, there is no epidemiological evidence of BCG-induced protection in HIV-infected children (168). A possible explanation for this lack of protection is an HIV-related suppression of the T cell-mediated response against infection and hematogenous spread of tubercle bacilli (376).

Studies of the protective efficacy of BCG against pulmonary TB reveal a striking variation from a negative effect to an efficacy of 80% in different populations and geographic regions (21, 377). Two large trials with apparently divergent results deserve particular attention: the British Medical Research Council (MRC) trial and the Chingleput trial in India.

The MRC trial from 1950 studied the protective efficacy of the Danish BCG strain in British school children aged 14–15 years (378). Subjects with prior mycobacterial sensitization were excluded after skin testing with a high concentration of tuberculin (100 TU of Old tuberculin). Follow-up was comprehensive, and after 15 years the overall protective efficacy was 78% and the efficacy against pulmonary TB was 77% (15). Interestingly, percutaneous administration of heat-killed *M. microti* studied in parallel to BCG provided equivalent protection.

The highly variable estimates of BCG efficacy against pulmonary TB found in a series of trials prompted what became the largest controlled BCG trial ever designed, starting in 1968 in the rural Chingleput district near Madras (379). 281 000 individuals aged one month or more were randomly allocated to vaccination with either BCG Danish, BCG Pasteur 1173 or placebo. The assessment of vaccine efficacy was based on individuals with a TST reaction of ≤7 mm to 3 TU of PPD-S, and only culture-confirmed cases of pulmonary TB were considered. Such cases are rare in children and consequently childhood forms of TB were not investigated. A paradoxical adverse effect of BCG was

demonstrated in vaccinated subjects during the first 5 years after vaccination: the TB incidence was nearly twice as high in those receiving BCG than in the non-vaccinated group (see p. 58). This difference was highly significant ($p<0.01$) (377). There was, in contrast, a 45% protective effect in the following 5 years in children ≤14 years at vaccination, whereas BCG had no effect in those aged ≥15 years from 5 years after vaccination and onwards (377, 380).

The effectiveness of the Swedish BCG vaccination program during the past decades has been evaluated by Romanus (12). Based on the observed increase of TB in children after 1975, the protective effect of the vaccine used in 1969 to 1974 was estimated to about 85%. The effectiveness of the following selective vaccination program was about 82%, as indicated by a declining incidence of TB in parallel with the increasing BCG coverage of the population at risk.

The maximum BCG efficacy level of around 80% is in the same range as the estimates of the protective effect of previously acquired tuberculous infection. That BCG vaccination can achieve a similar overall protective effect as natural tuberculous infection is illustrated by observations by Hyge of an outbreak of TB at a girls' school in Denmark (14): one group of 105 TST positive girls and another group of 106 BCG-vaccinated girls produced two cases of TB each, whereas 94 TST negative girls produced 41 cases – morbidity rates of 2%, 2% and 44% respectively.

Protection of adults

Evidence from randomized controlled trials on the efficacy of BCG vaccination is mainly derived from studies of infants and school children. However, several early controlled studies in HCWs and other adults from the United States and northern Europe indicate that primary vaccination of young adults is efficacious (reviewed in (17, 26)). In Sweden, a large study by Dahlström and Difs of conscripts with an average age of 21 years showed a 5-year protective efficacy of 63% (13). Due to the relatively high TB prevalence at the time, many conscripts developed TB before the protective effect of BCG could set in. According to Dahlström and Difs, full BCG protection would be expected after 6 months. If the TB cases that developed within 6 months of vaccination were subtracted, the protective efficacy was estimated to 77%. Dahlström later found that TB in BCG-vaccinated young adults has a milder course (381), and thus protection is not only a question of incidence but also of severity of disease.

Reasons for variability of protection

A meta-analysis of the over-all BCG efficacy (274) has received much attention, as it meets the general desire to simplify a complicated issue. The analysis concluded that BCG on average reduces the risk of TB by 50%. Such a conclusion is not only statistically improper (21) (due to the highly significant variation in efficacy against pulmonary TB between populations) but may also be misleading (21), as it implies that the observed variability in BCG efficacy is attributable to chance variations between studies and not by true differences between settings. As exemplified by Fine (21): "The implied logic is comparable to calculating the mean of the per capita incomes of Burkina Faso and of Switzerland and concluding that the world is, on average, middle class."

Possible reasons for the variable results of BCG studies in different settings are mentioned below.

BCG substrain variations

Although different protective effects are not proven, the apparently different qualities between BCG substrains could contribute to the variability in efficacy of BCG trials. On the other hand, the same substrains have shown markedly different protection in different trials (e.g. BCG Danish in the British MRC trial and the Chingleput trial), and different substrains (or vaccines) have shown very similar protection in some trials (e.g. BCG Pasteur 1173 and BCG Danish in Chingleput, and BCG Danish and heat-killed *M. microti* in the MRC trial). Thus, a major influence of different substrains on the variability in BCG efficacy is unlikely (382) and can certainly not explain all of the variation.

Genetic and nutritional differences

Host genetics as well as nutritional status of the vaccinees have been suggested to contribute to the variations in efficacy of BCG vaccination, but there is little evidence in support of these theories (21).

Exposure to non-tuberculous mycobacteria

As discussed in the previous section (on p. 31), infections with NTM can evoke varying degrees of protection against TB. Animal studies by Palmer and Long demonstrated that when BCG is superimposed on NTM infections, the resultant protective effect is only the same as that obtained with BCG alone (as opposed to the sum of both protective effects) (277). As stated by Palmer and Long: "All BCG can achieve is to fill the gap between what it

can do and what the atypical mycobacteria have already done." This "masking hypothesis" (353) implies that if subjects infected with NTM are not excluded from BCG trials, NTM-induced protection in the unvaccinated control group will reduce the observed protective efficacy of BCG to a corresponding degree (275).

Furthermore, recent studies in mice indicate that NTM infections can provide sufficient immunity to inhibit BCG replication, leading to reduced vaccine-induced protection (383). According to this "blocking hypothesis" (353), immunity induced by NTM is capable of blocking growth of low-virulent strains such as BCG, but not of more virulent strains such as experimental live vaccine strains (384, 385) or *M. tuberculosis.* As the "blocking hypothesis" implies less NTM-induced immunity against TB, it is more consistent with the high TB prevalence in tropical areas than the "masking hypothesis" (although it is possible that the TB prevalence in tropical areas would have been even higher without NTM-induced immunity (353)).

The randomized controlled BCG trials which showed the highest protective efficacies are those which most effectively excluded prior mycobacterial exposure (377): the MRC trial by skin-testing with 100 TU (378), a trial of North American Indians by skin-testing with 250 TU (386), and a trial of infants in Chicago by vaccinating soon after birth (387). In less successful studies the subjects were selected on the basis of a negative reaction to 5 TU or 10 TU of PPD-S (84, 388-390). For example, over 90% of the adults included in the Chingleput trial showed skin-test reactivity indicative of NTM infection (positive reactions to PPD-B) (84).

The results of the mentioned BCG trials as well as many other epidemiological studies of BCG-induced protection are consistent with a north-south gradient in the effectiveness of BCG, with poor efficacy in tropical and subtropical regions (353, 391). A corresponding gradient of NTM exposure is proposed as a plausible explanation to this phenomenon (274, 275, 353).

Previous exposure to Mycobacterium tuberculosis

The adverse effect of BCG in the first years of the Chingleput trial cannot be explained by the above mentioned factors. Furthermore, the consistency of this result over all age groups and its high statistical significance speak strongly against a chance finding. Springett and Sutherland suggested that subjects with LTBI who respond with weak DTH to the infection - and therefore would not be excluded from e.g. the Chingleput trial - may be at

increased risk of reactivation disease soon after vaccination (377). According to this hypothesis a BCG infection could intensify their cellular immune response, leading to exacerbation of disease. Such a phenomenon is in accordance with not only the early adverse effect in the Chingleput trial, but also with the low levels of efficacy observed in other trials. Interestingly, Dahlström found no adverse effect when vaccinating newly infected subjects in Sweden (381). This contradiction may be a consequence of differences between newly acquired and more remote infections. An alternative explanation is provided by differences between the settings in exposure to NTM and other agents such as helminths.

Helminth infections

Helminth infections such as filariasis and schistosomiasis are potent inducers of a Th2 response (392), as well as stimulators of regulatory T cell activity (393), and can thereby interfere with the expected Th1 response against BCG and other mycobacteria. Thus, BCG induced a Th2 response in a study of newborn infants who had been exposed *in uteri* to antigens from maternal helminth infections (394). In a study by Elias and co-workers (395), de-worming of helminth-exposed BCG-vaccinated individuals improved PPD-induced Th1 responses, and BCG vaccination boosted Th1 responses more in the treated group than in the placebo group. A subsequent larger study by the same group showed that an impaired BCG-induced Th1 response due to helminth infection was associated with enhanced production of the regulatory cytokine TGF-β, but not with Th2 activity (393). Although there is direct evidence of reduced BCG efficacy due to helminth infections in mice (396), corresponding evidence in humans is still lacking, and an ongoing study is investigating this possibility (397).

Interactions between NTM and helminth infections

Based on results from animal experiments and findings of large IL-4 responses in TB patients in developing countries, Rook and co-workers hypothesized that NTM infections in these countries may prime Th2 responses under the influence of helminth infections (392). Although the background Th1 activity protects against low-dose challenge with tubercle bacilli, they suggested that high-dose exposure may enhance pre-existing IL-4 activity that compromises cell-mediated immunity and leads to disease progression. According to this hypothesis, BCG fails in these populations since an effective vaccine needs to block the Th2 response rather than to strengthen the Th1 response as achieved by BCG vaccination.

Differences in progression from infection to disease

The hypothesis that a major action of BCG is to protect against hematogenous dissemination of tubercle bacilli implies that BCG would protect better against pulmonary disease due to endogenous reactivation than against disease attributable to exogenous reinfection (398). In areas where the probability of reinfection is high, the efficacy of BCG would then be lower than in areas where reactivation disease predominates. BCG efficacy would consequently depend on the TB prevalence in the population, but also on the virulence of the prevailing TB strains (see below). This hypothesis is consistent with data from the Chingleput trial, which showed a high risk of infection in the population, but a low incidence of TB among individuals who were initially TST negative, and a long interval between TST conversion and evidence of disease (398). However, the hypothesis is contradicted by the high BCG-related protection in North American Indian populations in which infection rates were very high (386), as well as by the MRC trial, in which BCG efficacy remained high in spite of a rapid decline in infection rate (15).

Differences between M. tuberculosis strains

Antigenic variation among *M. tuberculosis* strains may affect the immune response against tuberculous infection (399). Studies in guinea pigs showed that the *M. tuberculosis* strains from the Chingleput area were of low virulence (400) and could have an immunizing effect (380), suggesting the possible combination of masking of BCG efficacy with a relatively low risk of reactivation disease (380). In addition, Rhee and co-workers speculated that the extent of relatedness to BCG of the prevalent *M. tuberculosis* strains may be an important factor determining vaccine efficacy (401).

No consensus has been reached regarding these competing hypotheses. It is likely that several factors contribute to the observed variation in BCG efficacy.

Duration of protection

A review of 10 randomized controlled BCG trials found that the changes in effect over time varied considerably between trials (22). Based on a summary estimate of the BCG efficacy after 10 years that did not show any significant protection, the authors concluded that "there is no good evidence that BCG provides protection more than 10 years after vaccination". Analogous to the above-mentioned statement regarding over-all BCG efficacy (274) (see p. 57), such a conclusion does not include the possibility of true variations between settings. For example, there is very good evidence of longer protection in Great

Britain. An efficacy of 59% was demonstrated during the period from10 to15 years after vaccination in the MRC trial (15) (compared to an 83% efficacy during the first five years (402)). The TB incidence decreased substantially during the trial, and there were too few cases for a reliable assessment of efficacy after 15 years (278).

More recently, a 39% protection of neonatal BCG against all forms of TB lasting up to 20 years was demonstrated in children in Brazil (403). The longest demonstrated duration of BCG-induced protection was in a recent 60-year follow-up of a placebo-controlled trial among American Indians and Alaska Natives (404). The efficacy in the original study that started in 1935-38 was 75%, based on radiographically diagnosed TB at about 11 years (405). The follow-up study, encompassing the period from 1948 to 1998, revealed a protective efficacy of 52%. A slight but not statistically significant waning of the efficacy was observed during the follow-up.

Decreasing BCG efficacy may not only be caused by waning of BCG-induced protection in vaccinated subjects, but also by an increase in protection among those who are non-vaccinated, due to progressive exposure of the population to other immunizing infections. This is a likely contributing factor to a gradual decrease of protection in areas where NTM infections are common (275, 315).

REVACCINATION

Repeated vaccinations with BCG were previously standard in most national BCG programs. Many health authorities have chosen to discontinue revaccination in parallel with decreasing TB prevalence, but it is still extensively practiced in e.g. Eastern Europe and countries of the former Soviet Union (25). Given the absence of convincing evidence for the utility of repeated doses, the WHO has issued a statement discouraging revaccination (406).

The practice of revaccination is motivated by studies showing significant waning of BCG-related efficacy over the years (22). Revaccination has also been justified by the gradual waning of BCG-induced TST reactivity observed in many populations (141), based on the opinion that post-vaccinal TST reactivity is correlated to protection. One school of thought behind repeated BCG vaccinations in Eastern Europe claims that persistent BCG infection is a prerequisite for maintenance of protective immunity, and that loss of TST reactivity indicates elimination of the bacilli and the need for revaccination (289). The need for persistent BCG replication for continuous protection is supported by results from animal models (353). However, the extent to which such persistence is required in humans is uncertain, and the significance of

BCG-induced TST reactivity for protection against TB is a subject of debate (see p. 64).

The extensive use of repeated BCG vaccination contrasts the lack of solid evidence for its effectiveness. However, the documentation *against* revaccination has not been convincing either. There have been mainly observational studies regarding this issue. Analysis of routine data from Hungary showed a decline in incidence after revaccination was introduced (407), and data from Poland showed a higher TB incidence in those not receiving a second dose of BCG (408). There were, however, methodological problems in these two studies, as pointed out by the WHO (406). After the second dose of BCG was discontinued in Finland, the number of cases did not increase in the following cohorts of children compared to the earlier cohorts with revaccinated children (409). Although persuasive against a revaccination program in Finland, the number of cases in this study was too small for a general conclusion regarding revaccination (24). Finally, an observational study of children in Hong Kong (410) found no difference in TB incidence between participants and non-participants in a revaccination program.

There have been only two randomized controlled trials of the effectiveness of a second BCG vaccination against TB. First, a trial in Malawi, where a previous survey of primary vaccination showed no protection against TB (411), reported no protection of a second vaccination (412). This result is not transferrable to countries where primary vaccination is effective. Interestingly, the study showed a 50% protective efficacy of revaccination against leprosy, for which primary vaccination in Malawi has a corresponding protective effect (411). Second, a cluster-randomized trial of a second BCG vaccination in more than 200 000 school children in Brazil found no additional protection against TB in revaccinated children (413). The sample size in this trial was adjusted for the inclusion of children with LTBI, instead of attempting to identify such children by a TST (414).

A study of revaccination of Swedish adults showed a pronounced and persistent increase in the Th1 response against mycobacterial antigens, with a magnitude equal to that of primary vaccination **(IV)**. This increase in cell-mediated immunity is consistent with a protective immune response (see p. 66) to a second dose of BCG. Similar in vitro results were obtained in studies of adults in Japan and the US (415, 416), as well as in a study of revaccinated school children in Brazil during the mentioned BCG trial (417).

METHODS USED FOR ESTIMATING VACCINE-INDUCED PROTECTIVE IMMUNITY

For a long time the only available alternative to large epidemiological studies for assessing the human response to BCG vaccination were analyses of skin test reactivity to mycobacterial antigens and measurements of the post-vaccinal lesion or scar. Results from such studies are briefly reviewed below and their relevance for estimating protective immunity is discussed. Knowledge emerging from studies of the immune mechanisms involved in the control of mycobacterial infections has lead to new tools for analysis of the immune response to BCG. In the quest for an improved vaccine, such biomarkers could be used to assess the potential efficacy of vaccine candidates in relatively small clinical studies, before the launching of large-scale randomized controlled trials. An overview of some new potential correlates of protection follows below.

BCG Scar

In a mixed population of vaccinated and non-vaccinated individuals, the presence of a typical BCG scar is a marker of some protection against TB in countries where BCG is effective. Whether or not the presence (or size) of a scar is associated with protection *in BCG-vaccinated subjects* remains to be conclusively determined (21). In a paper on TB prevention in Moscow, Mitinskaya briefly reported strong correlations between presence and size of BCG scars and TB morbidity, but the study design and analyses were not described (418). A study in Malawi found no evidence of a relationship between scar size and protection against TB (367). However, the possibility for this study to detect such a relationship was small, as BCG confers no significant protection against TB in Malawi (411). Furthermore, the results suggested an *increased* risk of TB with increasing scar size, possibly because presence of LTBI at the time of vaccination resulted in larger scars and a higher subsequent risk of TB. Such a correlation could partially mask a possible association between scar size and vaccine-induced protection.

A study of Indian infants provides some evidence against a correlation between scar formation and TB protection: infants who did not develop a scar after vaccination had no reduction of cell-mediated immunity, as measured by PPD-induced leukocyte migration inhibition (419). Epidemiological data from Sweden points in the same direction. The low rate of scar formation observed in Swedish children **(II)** (340) is in contrast to the high concurrent effectiveness of the selective BCG vaccination program (420). Furthermore, Swedish subjects without a scar after BCG vaccination were TST positive more frequently than non-vaccinated subjects **(II)**, indicating that BCG produced a systemic reaction - as manifested by persistent DTH against tuberculin - in the absence of local

ulceration and scar formation. It has been suggested that ulceration of the lesion may fail to occur in spite of a local induration, and that bacilli may nonetheless multiply within the body and induce an immune response (25). An alternative explanation for absence of scars is the gradual disappearance of scars with time **(II)**, particularly after infant vaccination (323).

The tuberculin skin test

The significance of DTH in protective immunity against TB has been studied and discussed for decades. Whereas some authors have argued that the killing of infected macrophages by DTH is a prerequisite for resistance against TB (421), others have claimed that an ideal vaccine should not induce TST reactivity (422), since DTH is involved in the process leading to tissue destruction and pulmonary cavitation in TB (81, 423). The complexity of this issue is demonstrated by the papers by Dannenberg, some of which have emphasized detrimental (81) and some favorable (424) effects of DTH.

It is obvious that TST reactivity after BCG vaccination and resistance against TB often parallel each other. The assumption that TST conversion is a useful correlate of BCG-induced protection is strongly rooted in former literature (171, 349, 425). It has therefore been common practice for many years to interpret a positive TST in BCG-vaccinated individuals as an indicator of protection (5, 289, 426). However, evidence against this view has gradually accumulated over the years, and some contemporary authors claim that TST reactivity after BCG vaccination is not a correlate of protection (24, 422).

Although TST reactivity and protection against TB generally overlap, animal studies show that they are dissociable phenomena. It is possible to produce TST reactivity in guinea pigs without increasing resistance against challenge with tubercle bacilli (427), and it is possible to induce resistance without TST reactivity (428), as in experimental NTM infection (277). Furthermore, DTH and protective immunity to TB in mice can be selectively transferred by separate T cell populations (429).

Studies in guinea pigs show that waning and tuberculin-induced boosting of TST reactivity is not related to resistance (244). After demonstrating the booster phenomenon in BCG-vaccinated children, Guld and co-workers concluded that: “The very common practice of revaccinating on the basis of waning of tuberculin sensitivity, and of withholding vaccination from individuals who after tuberculin testing (…) fail to show waning of allergy, has therefore no scientific basis whatsoever…” (244). A following study by Nyboe showed that TST reactivity was increased after repeated vaccination of TST negative children as compared to primary vaccination (333). Likewise, repeated

vaccination of TST negative subjects are followed by a stronger and more rapid development of the post-vaccinal lesion compared to primary vaccinations (324, 333, 430). These signs of increased immune responses after repeated vaccination are indications of maintained immunological memory in the presence of waned TST reactivity. Furthermore, the development of the BCG lesion after revaccination is no different in individuals who do not become TST positive after primary vaccination compared to in those with a positive reaction that has waned (381).

There is little human epidemiological data to elucidate this issue. The only study that demonstrates a correlation between BCG-induced TST reactivity after BCG vaccination and resistance against TB is the one by Heimbeck on student nurses in Oslo 1927-1936 (8) (see p. 40). However, the vaccine quality was probably variable during the initial years of the study (431), the subjects were not randomized, and other methodological aspects of the study have been criticized (432). Such objections may explain why these results have been somewhat neglected in more recent literature (433).

In the British MRC trial (434), more than 7000 of the BCG-vaccinated children were tuberculin skin-tested within a year. Follow-up results after 10 years demonstrated that BCG-induced TST reactivity was not correlated to the subsequent risk of TB. Technical variations in the production of different batches of vaccine resulted in varying TST reactivity but had no significant influence on the protective efficacy

A review of controlled trials of BCG vaccination from different parts of the world showed no relationship between the average tuberculin conversion rate in each trial and the efficacy of the BCG strains (10). However, a tendency for a positive correlation of TST sensitivity and protection was found within each vaccine strain studied (10). Although this review is often used as evidence against a correlation, it leaves the possibility that a weak relationship may exist within certain settings.

In vitro correlates of protective immunity have been used to evaluate the TST in this context. The T cell proliferation and IFN-γ production induced by mycobacterial antigens (see below) were analyzed in Swedish BCG-vaccinated HCW without known TB exposure (**III**). The measured immune responses were significantly stronger in a group of TST positive subjects than in a matched group with TST negative reactions, indicating a stronger Th1 response in the TST positive group. These results are consistent with those of studies from the Unites States performed 3 months (435) and 1–3 yrs (296) after BCG vaccination. Furthermore, in vitro studies demonstrate that a systemic immune response may be present without skin test reactivity. Thus, antigen-specific

lymphocyte proliferation (436, 437) and IFN-γ production (438) are more sensitive indicators of T cell responses against mycobacterial antigens than the TST.

Although people with a positive TST due to LTBI have some protection against developing TB from reinfection (see p. 39), the tuberculous infection confers a risk of reactivated disease (see p. 38). BCG-vaccinated subjects with LTBI are at risk of reactivation as well. This is illustrated by a large case-control study of children BCG-vaccinated at birth in the high-prevalence setting of Hong Kong: a clear positive correlation between TST reactivity and subsequent TB was demonstrated (267). The study confirms that a positive TST reaction in settings of TB exposure signals a significant risk of TB also in BCG-vaccinated subjects.

TST reactivity is a dynamic process, influenced by a multitude of host factors, environmental factors and test-related factors (see p. 50). Consequently, the TST reactivity recorded at a given time point, e.g. soon after BCG vaccination, is more or less prone to change and may be different at a later occasion, as when the immune system is challenged by TB exposure. The possibility remains that TST reactivity at the time of exposure in BCG-vaccinated subjects is correlated to resistance against TB within specific settings. Such a correlation would be more difficult to demonstrate for the often labile TST reactions achieved by BCG vaccination than for the relatively persistent reactions due to LTBI. Considering the counter-evidence, however, a correlation is doubtful.

In vitro correlates

Acknowledging the insufficiency of the TST as a correlate of protection against TB, better correlates are much needed. The ability to stimulate T cell release of IFN-γ is at present the most extensively used surrogate marker of vaccine-derived protective immunity. The rationale behind this method is the critical role of IFN-γ in resistance against mycobacterial disease, in which Th1 cell-mediated activation of macrophages destroys intracellular tubercle bacilli. This key function of IFN-γ is clearly demonstrated by the susceptibility to mycobacterial infections of humans unable to produce or respond to IFN-γ due to mutations in genes involved in the Th1 cytokine pathway (371, 439).

Human in vitro studies suggest that antigen-specific IFN-γ responses vary with the protective immune response against TB. More specifically, *the increase* of IFN-γ production in peripheral blood or separated mononuclear cells, induced by stimulation *in vitro* with mycobacterial antigens such as PPD, has been shown to correlate with the known protection against TB induced by BCG in various human populations. Thus, BCG vaccination of Swedish young adults

induced a pronounced and persistent increase in PPD-induced IFN-γ responses **(IV)**. Ravn analyzed antigen-specific IFN-γ responses at different time-points following BCG vaccination of Danish subjects (279). IFN-γ responses increased more rapidly in subjects previously sensitized to mycobacterial antigens than in non-sensitized subjects, consistent with a memory T cell response induced by NTM. In contrast, a study from South India found that despite TST conversion after BCG vaccination, the PPD-induced IFN-γ response did not increase significantly (440), a finding consistent with the lack of BCG efficacy previously demonstrated in the same area (84). Studies of young subjects in Malawi and the United Kingdom also provide an illustration of contrasting IFN-γ responses in populations with different degrees of BCG-induced resistance (315): an increase of the PPD-induced IFN-γ response was seen after vaccination in the United Kingdom but not in Malawi.

Although the IFN-γ responses seem to correlate well with BCG-induced protection of the populations in these studies, it is also evident that the mechanisms of anti-tuberculous immunity are more complex than the effects of T cell-derived IFN-γ production. IFN-γ responses did not correlate with vaccine-induced resistance in recent experiments in mice (441-443) and results of recent human studies also question the reliability of IFN-γ responses as correlates of vaccine-induced protection. In the above-mentioned study of repeated BCG vaccination of school children in Brazil (417), the IFN-γ response increased significantly in half of the children, although revaccination provided no protection in the following evaluation of the trial (413). Furthermore, preliminary results from a study of South-African children BCG-vaccinated at birth indicate that antigen-specific IFN-γ levels 10 weeks after vaccination did not differentiate infants who would develop disease before two years of age from those who were resistant against TB exposure in the household (444).

Emerging evidence for important roles of CD8 cells, Th17 cells, γδ cells and regulatory T cells in TB defence (23) suggest that resistance against TB requires complex interactions between different T cell populations. Profiles of cytokine expression may therefore be more accurate as correlates of protection than IFN-γ levels only (444). An alternative strategy is to identify *correlates of susceptibility*, such as antigen-specific IL-4–producing CD8 cells and γδ cells (74, 77). The balance between opposing cytokines is probably crucial for latency and disease progression (79). Furthermore, in vitro "killing assays" may be used to evaluate the capacity of post-vaccinal T cells to kill intracellular mycobacteria, and inhibition of mycobacterial growth in whole blood can be measured by various methods (29).

PROTECTION AGAINST OTHER DISEASES THAN TUBERCULOSIS

Several studies suggest significant BCG-induced protection against mycobacterial diseases other than TB, analogous with NTM-induced heterologous immunity against TB. Furthermore, several epidemiological studies suggest a non-specific protective effect of BCG vaccination against various diseases.

Leprosy

BCG vaccination provides protection against leprosy to a varying degree. The efficacy ranges from 20-80% between different populations (411), a variability reminiscent of that found against TB. Studies in which protection against leprosy and TB were estimated in the same populations indicated better protection against leprosy (411, 445, 446) and, as previously mentioned, a protective effect of repeated vaccination was observed in Malawi against leprosy but not against TB (412).

Disease caused by non-tuberculous mycobacteria

Animal experiments have demonstrated that BCG vaccination can provide protection against NTM infections (447, 448). Epidemiological data support a corresponding effect also in humans. The discontinuation of BCG in Sweden was associated with a sharp increase in cervical lymphadenitis in children caused by NTM, in particular disease caused by *M. avium* (94). Although the observed increase may in part be explained by different forms of bias (94), the incidence of NTM lymphadenitis was considerably higher in non-vaccinated than vaccinated children during a subsequent 10-year period (1975-1985), and a decline was observed in children born to foreign parents in parallel with the increasing BCG coverage in this population (94). Observations in Finland (449) and the Czech Republic (450) support these findings.

Marked differences between countries in the incidence of disseminated NTM disease associated with AIDS have been attributed to different BCG coverage in the populations (451). Thus, in the 1980's the lifetime risk of NTM disease in AIDS patients was estimated to 50% in the Netherlands and 30% in the United States (both of which have very low BCG coverage) but only 10% in Sweden (in which BCG coverage was high). Finally, BCG-related protection against Buruli ulcer, a skin disease caused by *M. ulcerans* mainly seen in West Africa, was indicated by some studies (452, 453) but not by others (454).

Non-specific effects

BCG is a powerful immune stimulator. Its non-specific immunostimulatory potential is successfully utilized in immunotherapy for superficial bladder cancer (455). The tumour-cytotoxic effects are thought to occur by cytokine-mediated recruitment and stimulation of several lines of effector cells (455). Furthermore, BCG vaccination is claimed to confer non-specific preventive effects against several diseases in addition to TB and other mycobacterioses. Leukemia and other malignancies have been discussed in this context (456, 457). A long-lasting stimulation of Th1 responses has been suggested to prevent disease caused by helminth infections (458). Furthermore, a BCG-related decrease in the frequency of atopic manifestations has been shown in many studies. BCG and other mycobacteria can both prevent and diminish allergic responses in animal models by boosting either Th1 responses or allergen-specific regulatory T cells (459). Although several trials indicate a preventive potential, the effects of BCG vaccination on atopy in human trials remain controversial (459).

Recent large cohort studies in West Africa suggest a protective effect against overall mortality in infants, which cannot be explained by protection against TB (460-462). These effects have been attributed to a BCG-induced activation of the antigen-presenting ability of dendritic cells, leading to a general promotion of Th1 responses (313), but also to promotion of Th2 responses to vaccine antigens including hepatitis B and oral polio virus (463). Reduced mortality is more likely to occur in areas with high infant mortality rates than in high-income countries. Interestingly, the beneficial effects were often mostly pronounced among girls (462, 464, 465) and non-specific effects against mortality were also observed for measles vaccine (462). The validity of these observations has been questioned, e.g. due to possible confounding associated with the selective distribution of vaccines according to socio-economic status (460, 466).

Pros and cons of BCG

Pros

- Highly effective worldwide against disseminated forms of disease in infants
- Effective against pulmonary TB at northern latitudes
- Effectivity no less against multi-drug-resistant forms of TB
- Effective against leprosy
- Potential beneficial effects against other diseases
- Safe
- Low cost
- Scar as proof of vaccination

Cons

- Insufficient against pulmonary TB in areas close to the equator
- Waning protective effect
- Risk of disseminated BCG disease in HIV-infected infants and other immunodeficiencies
- Frequent mild adverse effects
- Difficult route of administration

AIMS

The overall aims of this thesis were to study the effects of BCG vaccination on the protective immune response against TB and to analyze the implications of TST reactivity and presence of a BCG scar in healthy subjects.

Specific aims were:

- to analyze the distribution of TST reactions among BCG-vaccinated and non-vaccinated healthy adults (**I**)
- to estimate the influence of various epidemiological factors on TST reactivity in healthy adults (**I**)
- to analyze the scar rate and the correlation between scar presence and TST reactivity in BCG-vaccinated children and adults (**II**)
- to analyze the association between TST reactivity and in vitro correlates of protective immunity in BCG-vaccinated adults (**III**)
- to study the protective immune response to BCG vaccination and revaccination in adults (**IV**)

DISCUSSION

I. Tuberculin skin test reactivity of young adults in a country with low prevalence of tuberculosis

Summary

The distribution of TST reactions among BCG-vaccinated and non-vaccinated healthy young adults was studied, and the influence of various epidemiological factors on TST reactivity was estimated. Significant TST reactions were frequent in BCG-vaccinated subjects, whereas most unvaccinated subjects were non-reactive. BCG vaccination, a country of birth with medium/high incidence of TB, and increasing age were strongly correlated to TST reactivity. Sensitin-dominant reactions were common in non-vaccinated subjects and were found in about half of those with TST reactivity.

Discussion

A strong influence of BCG vaccination on TST reactivity was demonstrated. The rate of 42% of TST reactions ≥10 mm in BCG-vaccinated subjects was higher than anticipated, considering the waning tendency of BCG-induced TST reactions. The influence of age, geographic origin and NTM infection was expected. NTM infections may explain much of the TST reactivity in BCG-

vaccinated subjects as well. The trend of increasing TST reactivity with age is probably related to increasing prevalence of NTM sensitization. The method used for detecting NTM infections is likely to provide a minimum estimate, considering that only half of the observed TST reactions in non-vaccinated Sweden-born subjects were sensitin-dominant in spite of the low risk of TB exposure. The TST results support 15 mm as an appropriate cut-off value in low-prevalence settings for a positive TST in healthy adults without known TB exposure.

II. BCG scar and tuberculin reactivity in children and adults

Summary

TST reactivity and the presence of a BCG scar were retrospectively analyzed in BCG-vaccinated children and adults. Most adults had a BCG scar, whereas a scar was identified in only half of the children. There was a strong positive correlation between scar presence and TST reactivity at all ages, particularly in small children. Furthermore, vaccinated subjects without a scar were TST positive more frequently than non-vaccinated subjects. In a prospective part of the study, vaccination of adults resulted in consistent scar formation.

Discussion

An unconfirmed history of adult BCG vaccination is probably incorrect if there is an absence of a BCG scar. The corresponding conclusion cannot be drawn from the absence of a BCG scar in a child. Furthermore, lack of scar formation after vaccination of a healthy adult suggests that the vaccination procedure is inadequate or the immune response is divergent.

Despite the low scar rate in children, the protective effectiveness of BCG in Sweden is high. Whether this paradox is due to a gradual disappearance of scars in children or the possibility of scar formation not being correlated to BCG-induced protection is not clear. Nevertheless, the utility of the BCG scar as an indicator of vaccine-derived protective immunity is doubtful. The TST results suggest that BCG can induce a systemic immune response without a persistent scar being formed.

III. The tuberculin skin test in relation to immunological in vitro reactions in BCG-vaccinated healthcare workers

Summary

The immune response in vitro against mycobacterial antigens was studied in BCG-vaccinated healthcare workers without known TB exposure. Lymphocyte proliferation and IFN-γ production of peripheral blood mononuclear cells were analyzed after stimulation *in vitro* with mycobacterial antigens. The immune response was significantly stronger in a group of TST positive subjects than in a matched group with TST negative reactions.

Discussion

The study demonstrates a stronger Th1 response after BCG vaccination in a group of TST positive subjects than in a TST negative group. The results are consistent with those of BCG-vaccinated adults in the United States and support a correlation between TST reactivity and a protective immune response in BCG-vaccinated populations without known TB exposure.

Subjects with large positive TST reactions were chosen in order to increase the probability of detecting a difference in Th1 responses in a relatively small sample of subjects. Thus, the study does not provide information of the Th1 response in subjects with moderately positive reactions.

IFN-γ has a decisive role in the Th1 response and in resistance against TB. T cell release of IFN-γ is at present the most extensively used correlate of vaccine-induced protective immunity. However, protective immunity against TB is more complex than the effects of T cell-derived IFN-γ production. It is therefore uncertain if the demonstrated differences in IFN-γ production *in vitro* reflect true differences in protective immunity.

IV. Primary vaccination and revaccination of young adults with BCG: a study using immunological markers

Summary

The immune response induced by BCG vaccination was analyzed after primary-vaccination and revaccination of TST negative young adults. PPD-induced lymphocyte proliferation and cytokine production of peripheral blood mononuclear cells were studied before vaccination, two months after vaccination and after one year. In the primary-vaccinated, as well as the

revaccinated group, lymphocyte proliferation and IFN-γ production increased significantly after two months and the increase was maintained after one year.

Discussion

Both primary vaccination and revaccination evoked a pronounced and persistent increase in the Th1 response. These findings are in line with in vitro results of other studies, but the high level of persistence after revaccination has not been reported elsewhere. Since primary BCG vaccination of adults is well documented in countries at northern latitudes, the immune response in the primary-vaccinated group was anticipated. The corresponding results for revaccination suggest a protective effect equal to that of primary vaccination. This result was less expected, considering the lack of conclusive evidence for the effectiveness of repeated vaccinations.

CONCLUSIONS

The influence of BCG vaccination and NTM infections on TST reactivity is considerable in our setting with low prevalence of TB. Consequently, the TST has poor specificity as an indicator of tuberculous infection in individuals without known TB exposure.

Absence of a scar after BCG vaccination is common in Swedish children, whereas vaccination of adults results in consistent scar formation. There is a close association between scar presence and TST reactivity. The TST results suggest that BCG can induce a systemic immune response in the absence of scar formation.

BCG-induced TST reactivity is related to in vitro correlates of protective immunity in our setting. Both primary vaccination and repeated vaccination of adults induced an in vitro response indicating enhanced protective immunity. The accuracy of the in vitro methods needs further evaluation.

The majority of healthy individuals exposed to *M. tuberculosis* will not develop active TB. Unraveling the mechanisms behind resistance against disease is a major challenge in TB research today. Identification of reliable in vitro correlates of protective immunity may facilitate the development of an improved vaccine.

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