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Introduction

In 1890, some eight years after identifying the tubercle bacillus as the causative agent of tuberculosis, Robert Koch delivered a lecture at the Tenth International Congress of Medicine in Berlin, in which he described the biological properties of a "brownish transparent fluid" he had prepared from *Mycobacterium tuberculosis* (1). Koch believed this extract, which came to be known as tuberculin (and later as Old Tuberculin), to have both diagnostic and therapeutic activities. The report immediately became the center of a whirlwind of attention and controversy. By the following year, the experiences with thousands of patients (treated with tens of thousands of doses) made it clear that tuberculin had no role in the treatment of pulmonary tuberculosis (2). However, Koch's observations regarding the differential responses of tuberculous and non-tuberculous subjects following injection with tuberculin proved remarkably insightful, and provided the impetus for countless studies to identify the antigens involved and determine the clinical significance of such reactivity. Indeed, 100 years later, skin testing remains the only means to identify individuals with clinically latent *M. tuberculosis* infection.

Purified Protein Derivative (PPD)

The tuberculin that Koch described was a mixture with glycerin of the filtered spent culture medium of *M. tuberculosis*. In 1931, Florence Seibert, a biochemist at the University of Pennsylvania, began a series of studies to identify the active principal of Koch's tuberculin. These experiments ultimately lead to the recognition that proteins, rather than polysaccharides or glycolipids, were responsible for skin test reactivity (3). The precipitation of these proteins from heated cultures of *M. tuberculosis* resulted in a reagent (purified protein derivative or PPD-S) with increased skin test reactivity and decreased febrile responses. A portion of one batch of PPD from these early studies remains today at the United States Bureau of Standards and the World Health Organization as the reference standard to which all current lots are compared.

Despite its name, however, PPD is not "purified" in any modern sense. Separation of PPD by gel electrophoresis or column chromatography reveals a nearly continuous slurry of proteins of small size with few discreet bands. This represents both the antigenic complexity of the secreted antigens of *M. tuberculosis*, and the partial hydrolysis of proteins due to heating. The fragments are no longer suitable targets for recognition by antibody, but remain readily recognized by sensitized lymphocytes. The mixture clearly contains both *M. tuberculosis*-restricted and cross-reacting antigens. Its complexity has confounded investigators interested in developing tests with greater specificity for *M. tuberculosis*.

Skin test placement and measurement of reaction size

Tuberculin skin testing is best performed by the Mantoux method, using PPD that has been stabilized with a detergent to prevent loss of reactivity to the wall of the glass vial. The vial should be refrigerated between uses. A beveled 25 or 26 ga. needle is

placed intracutaneously and 0.1 mL of solution injected on the volar surface of the forearm. Production of a raised, blanched bleb or wheal is essential. Deeper injections will result in dispersion of the antigen through the circulation, and will lead to a reduced or absent response. The reaction is read as millimeters of induration at 48-72 hr. This is best accomplished by through the use of ball-point pen and ruler. The pen is advanced toward the center of the indurated area until resistance is felt. This is repeated at successive 90° angles. The larger of the two distances between pen marks is recorded as the reaction size.

Some individuals will have an immediate response, manifested as edema, erythema, and pruritis, that is apparently antibody-mediated. Such reactions usually subside within 24 hr and are disregarded. Occasional individuals will have a delayed response (at 96-120 hr) that otherwise has the typical characteristics of positive skin test. The significance of such reactions is not certain, but most such individuals will have positive responses on retesting, and are usually considered positive.

Cross reactivity: The problem of "no-lesion" cattle

The landmark studies showing the clinical significance of tuberculin skin test reactivity were first performed in cattle, a species in which the clinical diagnosis of tuberculosis is difficult until very late in the disease. Several veterinary studies performed within a few years of Koch's original description of tuberculin showed that, after slaughter, tuberculin positive animals had a strikingly high frequency of mycobacterial lesions on autopsy (4, 5). The public health implications of these findings were clearly apparent. In the United States, routine testing was instituted on a mass scale, such that by 1910, over 200,000 tests were performed in Wisconsin alone. These campaigns led to a progressive decline in the rates of bovine tuberculosis during the early decades of the twentieth century in the US.

The success of the campaigns to eradicate bovine TB lead to the first recognition of one of the shortcomings of tuberculin skin testing, that of species cross reactivity. As the rate of bovine tuberculosis decreased, the proportion of tuberculin-positive animals without demonstrable lesions on autopsy progressively increased (6). These "no-lesion" cattle were found in herds with low numbers of lesion-positive animals and low numbers of reactors generally. These epidemiological findings suggested that tuberculin sensitization could occur via infection with organisms other than *M. tuberculosis*, and that these organisms were not readily transmitted from one animal to another. Subsequent studies showed that animals could be readily sensitized to tuberculin by infection with non-tuberculous mycobacteria such as *M. avium* and rapidly growing species (7-9). Although the largest reaction sizes were observed in animals tested with a reagent prepared from the same strain as that used for sensitization, the species specificity of the reaction was clearly relative and not absolute.

Skin testing in BCG vaccinated individuals

Vaccination against tuberculosis with *M. bovis* BCG is performed in infancy in over 100 countries worldwide. In some countries, vaccination is repeated when children enter public school. Vaccination typically results in a small raised, hypopigmented 3 mm papule at the vaccination site, usually the upper arm. Larger scars occur in individuals with prior PPD skin test reactivity; scars often do not occur when the vaccine is administered to HIV infected infants. BCG scars are readily recognized within the first few years after vaccination, but recognition may become problematic over time (10, 11).

BCG is not administered in the US, primarily because of the concern that it interferes with tuberculin skin testing. Indeed, a median reaction diameter of 16.5 mm

was reported in healthy, PPD skin test negative adults in the US one month after vaccination (12). However, this response is relatively short lived, such that by one year, it had decreased to 9.5 mm. Skin test reactions in Ugandan children vaccinated at birth, without recognized exposure to tuberculosis in immediate family members, range from 3-5 mm through age 5 (C. Whalen, CWRU, unpublished observations). The reduced reaction size as compared to US adult vaccinees may be due age-related differences in immune function, different in vaccine strains, concomitant parasitic infection, or genetic factors. In any case, these data suggest that individuals from a high TB prevalence country who have PPD reactions of 10 mm or greater should be considered *M. tuberculosis* infected, unless there is compelling evidence of recent and/or multiple vaccinations with BCG.

Reaction size and subsequent risk of tuberculosis

Reaction sizes to a 5 TU *M. tuberculosis* PPD skin test (the bioequivalent of 0.1 μg) in individuals infected with *M. tuberculosis* are symmetrically distributed about a modal value of 16-17 mm. Reactions in individuals infected with *M. bovis* tend to be somewhat larger, but those due to most other mycobacteria are smaller (13, 14). Individuals with smaller diameter reactions (with a modal value of 4-5 mm diameter) are observed with increased frequency in the southeastern US. This corresponds to the region of the US with the highest rate of skin test reactivity to the *M. avium-intracellulare* complex (PPD-B, named for its site of isolation, Battey, GA). As a consequence, reactions of <5 mm are not considered positive, as in most cases, these are thought to be due to infection with other mycobacteria.

In most of the US, infection with *M. bovis* (or vaccination with BCG) is uncommon. As a consequence, reactions of 20 mm or greater are nearly always the result of infection with *M. tuberculosis.* As the reaction size decreases, the likelihood of non-tuberculous infection increases, and the risk of mycobacterial disease decreases due to the greatly decreased virulence of these organisms for otherwise normal hosts. Thus, the risk of subsequent tuberculosis is directly related to the reaction size. This relationship has been described in several diverse populations, in both high and low prevalence areas (15-18). The increased risk of tuberculosis in individuals with large reactions may be as much 10 fold greater than those with small reactions (18). Thus, the greater significance attributed to large reactions by many clinicians is quite appropriate.

The "booster phenomenon"

Several studies have documented that the proportion of individuals with positive tuberculin skin tests rises with repeated testing. This has become known as the booster phenomenon. Boosting may occur in the absence of recognized TB exposure, or less commonly, it may occur due to the waning of the immune response over time following infection with *M. tuberculosis,* due to aging or to progressive HIV infection (19). It is believed to be due to increased recruitment and expansion of antigen-specific lymphocytes in donors with initially weak reactions. Both the reaction size and the proportion positive have been observed to increase. The phenomenon may occur in tests separated by as little as 1 week, or as much as 6-12 months, but boosted responses do not persist for greater intervals in the absence of repeated exposure (20). Although boosting is recognized to occur in subjects whose responses to *M. tuberculosis* were initially positive but have declined over time, it appears much more commonly in individuals originally sensitized to non-tuberculous mycobacteria (21). For example, in one study, 13 of 14 apparent conversions on repeated PPD-B skin testing in 213 young, healthy mid-western volunteers occurred among those 103 subjects who initially had

positive responses to PPD-G, PPD-Y, or PPD-B (22). Only 1 occurred among the 110 individuals without non-tuberculous reactivity.

In populations in which infection with *M. tuberculosis* is less common than that with non-tuberculous mycobacteria, the booster effect will lead to an over estimation of rates of new *M. tuberculosis* infection. This is most problematic in individuals who are repeatedly tested as a part of a regular screening program, such as hospital employees or nursing home residents. Although boosting has been reported on the third or even fourth test of previously negative subjects, it occurs most often with the second test. For this reason, it is recommended that individuals beginning testing on a regular basis be tested a second time, one to two weeks after an initial negative test. The first test should be considered the true initial response. The second test should be used as a baseline value when evaluating possible future conversions.

The 250 TU ("second strength") skin test

Tuberculin skin test reagents of 250 TU (sometimes referred to as "second strength") have been studied extensively in the US, although their continued availability is uncertain at present. This reagent has 50 times the protein content of the 5 TU test, but is not standardized in terms of antigenic reactivity. The 250 TU test is occasionally used in cases in which *M. tuberculosis* infection is strongly suspected, but the 5 TU test is negative. The extent of cross-species reactivity is profoundly affected by the dose. As a consequence, positive reactions to a 250 TU test are observed in a high proportion of healthy individuals without a history of exposure to *M. tuberculosis,* particularly in the southeastern states of the US (table 1, reference (23)). Indeed, with increasing doses of PPD of up to 1 mg, reactivity can be demonstrated in up to 72% of infants with no known exposure history (24). For this reason, the 250 TU test is only potentially useful if negative, and even this of questionable significance in individuals with active disease.

Anergy in tuberculosis: Selective vs. general

Tuberculin skin test reaction sizes of patient with active pulmonary tuberculosis are smaller than those of healthy tuberculin reactors. A minority of TB patients will be classified as non-reactors. Several factors are recognized to result in reduced skin test reactivity in TB patients in an antigen-nonspecific fashion, including protein malnutrition, and deficiencies of specific micronutrients (25). In a study by Nash, 49 of 200 (25%) of patients with pulmonary tuberculosis had reactions of <10 mm to 5 TU PPD (26). Nineteen (10%) failed to respond to a 250 TU test. Non-responders showed decreased lymphocyte transformation in vitro to PPD. The hyporesponsiveness to PPD appeared primarily to be selective, in that all but 2 subjects responded to other non-mycobacterial skin test antigens. Thus, the use of a panel of control antigens does not appear to aid in the interpretation of skin testing in patients with tuberculosis.

Curiously, the specific hyporesponsiveness to mycobacterial antigens in tuberculosis appears to be mediated by monocytes. Monocyte activation is central to the immunopathology of tuberculosis, particularly in terms of over-production of the cytokines TNFα, transforming growth factor (TGF) β, and IL-10 (27). It is the excess production of the latter two of these cytokines that appears to mediate suppression of *M. tuberculosis*-induced lymphocyte proliferation in tuberculosis. Several specific mycobacterial constituents, including proteins, and the glycolipid lipoarabinomannan, stimulate production of these and other modulatory cytokines (28-30). The partial purification of PPD is not sufficient to remove the components leading to production of these suppressive factors. One of the goals of current TB skin test research is therefore

to determine whether selective removal of some of these factors can improve the sensitivity of skin testing in patients with active disease.

Stability of skin test reactions over time

At one time, it was believed that tuberculin reactivity remained stable over a lifetime for most reactors. This now is known not to be true. The process of transition from a positive to negative test is called reversion. Reversion may occur for several reasons, including impaired lymphocyte function due to aging, and decreased numbers of circulating CD4 T lymphocytes due to progressive HIV infection. It may also occur due to lack of continued antigenic stimulation in individuals in whom *M. tuberculosis* has been eradicated, either because of a protective immune response or because of preventive therapy. The frequency of reversion varies considerably in different populations. Havlir found that only 3 of 22 skin test reactors individuals originally identified as household contacts of TB cases had lost their reactivity when retested 19 years later (31). However, in the elderly, the proportion of reverters may be as high as 25% over a 3 year period (20). In that report, reversion was closely associated with the presence of other illnesses such as malignancy, and carried a poor prognosis due to the concomitant illnesses. Reversion also appears to occur with greater frequency in individuals with borderline reaction diameters, and in HIV-infected persons.

The effect of HIV infection on skin test reactivity

Infection with the human immunodeficiency virus type 1 (HIV-1) results in decreased cell mediated immunity, which is reflected in an increased risk of tuberculosis and decreased tuberculin skin test reactivity. Responses are generally preserved in patients with CD4 cell counts >200/µL, but are decreased in size and frequency in subjects with counts of from 100-200/µL, and are usually absent when CD4 counts drop below 200 (32, 33). The declining sensitivity of skin testing in this population can be offset if a threshold of 5 mm is used for identifying reactors. The lower threshold for HIV-infected persons results in equal proportions of reactors in HIV- and HIV+ persons from the same geographic region (34). Several studies have shown an increased risk of tuberculosis in PPD+ HIV infected persons. Identification of such individuals is clearly a priority, since it has been well documented that preventive therapy is effective in reducing the risk of TB in this group (35, 36).

Management of HIV-infected tuberculin negative individuals is less straight-forward due to the increased false negative rate of skin testing. The risk of tuberculosis in this group is uncertain, and may depend on the local prevalence of tuberculosis, and the degree of immunocompromise. Two studies have suggested that anergic subjects in particular may be at equal or greater risk than tuberculin reactors (37, 38). It has therefore been suggested that skin testing with a panel of microbial antigens, particularly mumps and candida, may aid in identifying individuals with profoundly impaired cell mediated immunity (39). However, several observations have led to a modification of this recommendation. Mumps reactivity may remain after loss of PPD reactivity in HIV-infected persons (40). In certain populations where TB is prevalent, skin test reactivity to PPD may be greater than that to several other antigens (41, 42). Even in populations where PPD reactivity is low, some HIV-infected individuals respond to PPD but not other microbial antigens (37). PPD-induced responses may be suppressed in a specific fashion in HIV-infected patients with active tuberculosis. Finally, the efficacy of TB preventive therapy in anergic individuals appears to be substantially reduced as compared to tuberculin reactors (35). This may in part be due to an increased role for

acquisition of new infection in these individuals, rather than reactivation of latent infection. This casts doubt on the importance of identifying such individuals.

Conversion to positive PPD reactions in the absence of TB re-exposure also appears to be more common in HIV infection. When 139 anergic HIV-infected Ugandans (candida and PPD reactions of 0 mm) were re-tested after 1.5 years, nearly half had regained reactivity to either antigen despite the absence of specific HIV therapy (J. Johnson, CWRU, unpublished observations). French et al. have reported transient PPD conversion in HIV-infected individuals without recognized TB exposure following initiation of therapy with zidovudine (43). This was thought to be due to restored cross-reactivity to *M. avium*. The effects of combination HIV therapy with protease inhibitors remain to be determined, but it is likely that skin test conversions will be observed with increased frequency here as well.

For these reasons, the US Centers for Disease Control now suggests an optional, adjunctive role for anergy panel testing (44). For physicians electing to perform such testing, the Mantoux (intracutaneous) is suggested, using FDA-approved mumps and candida antigens. Although a multiple puncture device has been approved for simultaneous testing to several antigens, there is less experience with such a format. The CDC panel noted that factors other than anergy, such as CD4 cell count and area of residence, may be more significant determinants of the risk of TB in HIV+ PPD- individuals (37).

Future prospects

The development of improved diagnostics for both latent and active tuberculosis is currently an area of great interest, both with respect to the antigens and the method for detection of reactivity. Several antigens among those expressed by *M. tuberculosis* appear to contain epitopes restricted to that species; of these, the gene for one, ESAT-6, has been deleted from most strains of BCG (45). It may be uniquely suited for specific detection of *M. tuberculosis* reactivity in BCG-vaccinated individuals. Removal of LAM and other immunosuppressive lipids from PPD may result in increased reactivity, particularly in individuals with active disease. Lastly, cytokine responses to these antigens can be detected *in vitro*, rather than measuring DTH reactivity (46, 47). Antigen-induced production of interferon-γ in cultured heparinized blood may more accurately reflect the adequacy of host defenses against *M. tuberculosis*, as this cytokine is a critical component of host defenses against intracellular infection. This approach has the added advantage in that a second visit for skin test reading is not required.

In summary, measurement of skin test reactivity to a partially purified extract of *M. tuberculosis* remains a key diagnostic tool for detection of asymptomatic infection with this organism. In the century that has elapsed since the discovery of this phenomenon, the methodology has been modified somewhat but not fundamentally altered. The future role of skin testing may be more profoundly changed during the coming decades, as species-specific antigens are identified, and as better tools to measure host immunity are evaluated in populations at risk.

Tables.

Table 1. Tuberculin reactivity in 16000 student nurses, according to residence, history of contact with tuberculosis, and PPD dose, from Palmer (23).

	contact with tuberculosis		
	none	intermediate	close
Proportion positive to 5 TU			
Northern and western states	.10	.19	.41
Southeastern states	.12	.19	.44
Proportion positive to 250 TU among those negative to 5TU			
Northern and western states	.29	.29	.27
Southeastern states	.67	.69	.70

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