

CURRENT STATE OF RESEARCH INTO SURROGATE MARKERS/CORRELATES OF PROTECTION FOLLOWING IMMUNISATION AGAINST TUBERCULOSIS

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SUMMARY

A correlate of protection is a laboratory parameter that has been shown to be associated with protection from clinical disease. Whole-blood production of interferon-gamma has been described as 'the flawed current gold standard' correlate. There are good reasons for and against using it. Suggestions have been made for improving this assay, and numerous proposals for alternative correlates have been made. Determination of true surrogate markers of protective TB immunity will require further knowledge of the specific human mechanisms of killing of intracellular *Mycobacterium tuberculosis* and TB-specific regional lung immunity. Lymphoproliferation assays and assays of mycobacterial killing potentially measure the most biologically relevant correlate of protective immunity induced by candidate TB vaccines. Recent advances in DNA chip technology should facilitate global analyses of the host responses to TB which will provide information on the correlates and surrogates of susceptibility and resistance as well as of protection. In current work being conducted in an area of high TB prevalence in the Boland, Western Cape, a novel approach is being employed to identify a correlate in a case control study. The primary putative correlate in this study is a whole-blood intracellular cytokine assay which detects the frequency of mycobacteria-specific T cells.

BACKGROUND

A correlate of protection is defined by the Food and Drug Administration as a laboratory parameter that has been shown from adequate and well-controlled trials to be associated with protection from clinical disease.¹ It is the ultimate aim of vaccination to produce long-lived immunological protection against pathogens by the production of a pool of memory cells. Vaccines such as smallpox, tetanus, diphtheria, yellow fever, pertussis, *Haemophilus influenzae* type B, poliomyelitis, measles, mumps and rubella have vaccination programmes aimed at producing high titres of antibody and are not aimed at cellular immunity. Esser *et al.*² suggest that these successful vaccines do not protect individuals from infection but rather against disease by giving the immune system a 'head start'; by inducing an antibody response that is effective against microbes that are antigenically stable and cause acute or lytic infections, not chronic infections. Further they maintain that understanding the mechanisms of cellular immunity is driven

by the need to develop vaccines against persistent or chronic pathogens such as HIV and TB. TB is particularly challenging as its intracellular location shields it from antibodies and a variety of T-cell subpopulations must be activated to challenge its antimicrobial defences.³

BCG is currently the only vaccine available for prevention of human disease caused by mycobacteria.⁴ The World Health Organization recommends *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) vaccination in areas of high TB prevalence, which includes South Africa. The clinical and immune effects of BCG are unclear.⁴ However the majority of healthy individuals exposed to *M. tuberculosis* will not develop disease and differentiating latent infection from disease poses a great challenge; the controversy regarding correlates of immunity persists because identifying infected but healthy individuals (those who are immune) has been problematic.⁵ The protective efficacy of BCG against pulmonary TB is controversial. One meta-analysis found that it can reduce the risk of pulmonary TB by 50% and TB-related death by 71%.⁶ It also varies geographically from no apparent protection in Malawi to 50-80% protection in the UK.⁷ It is likely that differential sensitisation due to exposure to environmental mycobacteria is the most important determinant of the observed differences in protection by BCG between populations.⁶ It is also possible that the relatively poor induction of immunity directed against secreted mycobacterial components may have contributed to the variable efficacy rates reported in previous BCG vaccine trials.⁶

Variables that affect the efficacy of the immune response to vaccination are age of the individual, exposure, route, and specificity and/or type of antigen. The incidence of TB in South Africa is in the region of 0.5% per annum; a striking feature of age-specific incidence rates is the burden of infection carried by adolescents (Fig. 1). In primary immunisation a correlate may be more easily defined than in adolescents or adults as exposure to TB and/or environmental mycobacteria occurs. *M. tuberculosis* is an intracellular organism that actively evades the immune system by inhibiting formation of phagolysosomes.⁸ Because the mechanisms used by human cells to inhibit intracellular *M. tuberculosis* are uncertain, true surrogate markers for protective TB immunity are not known.⁹

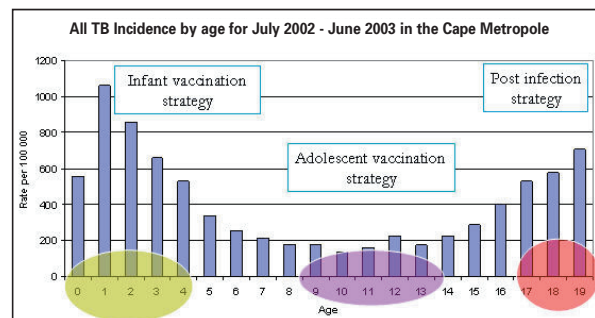


Fig. 1. Age-specific incidence of tuberculosis in Cape Town showing different vaccination strategies (Acknowledgments: Dr Hassan Mahomed, University of Cape Town).

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Correlates of protective immunity to *M. tuberculosis* in humans are desirable for:

1. Rational design of novel TB vaccines^{5, 10, 11} - research on improved TB vaccines is being hampered by the absence of a correlate of protective immunity in humans.^{7, 12} In most TB vaccine trials, vaccine efficacy has been measured by the prevention of clinically apparent disease. Diagnostic tests are centred on laboratory confirmation of a physician's suspicion of TB; this can be problematic, especially in childhood TB. Infection can lead to active disease or latent infection, so measurements of clinical disease alone may miss important information about the generation of protective immunity which might be useful in designing second-generation vaccines.¹³
2. Demonstrating the immunogenicity of a vaccine candidate and its potential efficacy.¹² To aid in the evaluation of pre-exposure and postinfection vaccines, tests are needed that can clearly and timeously distinguish immunological protection from vaccine failure.¹³
3. Optimising the dose, vehicle, adjuvant, and schedule of immunisation.¹²

THE NEED FOR CORRELATES FOR CONDUCTING TB VACCINE TRIALS

Research is necessary to identify surrogate markers of infection, disease, or protection to avoid the need to use clinically apparent disease as the endpoint in the vaccine trials.¹³ The availability of correlates of protective immunity in humans may be critical for efficacy trials of any new TB vaccine. Efficacy trials of BCG vaccine have had to enrol huge cohorts of subjects and follow them up for long periods.^{9, 12} This is a disincentive to commercial interest in TB vaccine development as such studies are difficult to perform, unlikely to be successful and extremely expensive to fund. It is likely to limit the number of candidate vaccines that can be tested in humans.⁹ By using correlates of protection as surrogate endpoints for vaccine immunological activity and efficacy, alternative, simpler and cheaper study designs could be developed. Surrogate markers of vaccine efficacy could also help identify which vaccine candidates have the most promise for more extensive studies.⁹

STRATEGIES FOR IDENTIFYING CORRELATES

Potential correlates can be proposed on the basis of animal models and *ex vivo/in vitro* studies in humans.¹² Ultimate validation will require correlation with protection in a phase III efficacy trial of an effective vaccine.¹² Promising correlates identified by other approaches can be selected for inclusion in phase I - III vaccine trials.¹² A number of approaches, each of which has attendant problems, may be taken, including:

1. animal models
2. human TB studies
3. household contact studies
4. immunogenetics
5. immunotherapy trials.

In current work being conducted in an area of high TB prevalence in the Boland, Western Cape, a novel approach is being employed to identify a correlate in a case-control study.¹⁴ Infants vaccinated with Tokyo 172 BCG at birth are bled 8-14 weeks post vaccination and their cells are banked. Participants are followed up for a minimum of 2 years post vaccination, and TB cases are identified and further blood products cryopreserved together with those of age- and area-matched controls. The primary putative correlate in this study is a whole-blood intracellular cytokine assay which detects the fre-

quency of mycobacteria-specific T cells, but the study has capacity to look at other candidate correlates as well.

All potential correlates need to be studied in phase I/II trials of vaccines to demonstrate that they are modulated by the vaccine under study before they are incorporated in the design of a phase III trial.¹² Correlates of protection are not necessarily part of the mechanism of direct protection. A new vaccine may induce protective immunity without engaging the pathways constituting 'natural' protection. Selection of correlates will have to be decided by reviewing the currently available data at the time a vaccine trial is about to start.¹²

Recognition of the *M. tuberculosis* virulence factor, early secretory antigenic target 6 (ESAT-6), may be used to identify healthy, but infected individuals. Signals for cytokines expressed directly *ex vivo* may then be compared with the pattern found in TB patients.⁵ Tuberculous pleuritis provides a good model to study the correlates of protective immune response at the site of infection, e.g. by studying the cell subset profile and cytokine assay in plasma and pleural fluid of TB patients.¹⁵ Humoral immune responses against a range of mycobacterial antigens have been examined in cattle.¹⁶ Whole-blood culture may be used to measure immunity to *M. tuberculosis*.¹⁰

BCG SCAR

BCG vaccine scarring has been used as a surrogate marker of vaccination or of effective vaccination.¹⁷ Jason *et al.*¹⁷ found no overall associations between the degree of BCG vaccination scarring and HIV serostatus, viral load, or the percentages of lymphocytes expressing CD4. The presence of BCG scarring does not imply that a long-term type 1 response pattern to mycobacteria has been conferred by vaccination.¹⁷ In infants, a BCG lesion (scarring or inflammation) was found to be associated with a diminished pro-inflammatory and interleukin-4 (IL-4) cytokine profile and lower rates of bloodstream infection and sepsis symptoms, suggesting an early, non-*M. tuberculosis*-specific clinically protective effect of BCG vaccination.¹⁷

DELAYED TYPE HYPERSENSITIVITY (DTH)

It has been shown that BCG-induced DTH does not correlate with vaccine-induced protection against TB, either between vaccines, between populations or between individuals.⁷ Several vaccine trials have demonstrated that a positive skin test in response to vaccination with BCG is not predictive of protection from TB.¹³ Many persons without purified protein derivative (PPD) skin test evidence of specific immunity to *M. tuberculosis* or known TB exposure, nevertheless display *in vitro* responsiveness to live *M. tuberculosis* and its antigens.⁹ The tuberculin skin test (TST) cannot readily distinguish BCG-vaccinated individuals from persons infected with *M. tuberculosis*.¹³

Black *et al.*⁷ found that BCG was associated with greater increases in both interferon-gamma (IFN- γ) and DTH responsiveness in the UK than in Malawi. The association between IFN- γ and DTH was weakened by BCG vaccination. They postulated that this provided evidence of a dissociation between cellular immunity and DTH in humans and that vaccine-induced changes in IFN- γ responses to mycobacterial antigens provide a better indicator of vaccine-induced protection than do effects on DTH.

PPD persistence and boosting after BCG vaccination are partially correlated with the induction of mycobacterial-specific lymphoproliferative and IFN- γ responses.¹⁸ Future studies are needed to further investigate the hypothesis that persons developing both persistent

DTH and type 1 immune responses after TB vaccination have increased protective mycobacterial immunity.¹⁸

CELLULAR IMMUNITY – THE T-CELL RESPONSE

More than 200 years after Edward Jenner provided the first scientific rationale for vaccination by demonstrating that individuals immunised with cowpox were protected from the disease caused by the smallpox virus, recent work¹⁹ has demonstrated residual immunity to smallpox virus up to 25 years after vaccination. There are three phases of the T-cell immune response; expansion, contraction and memory; the amount and time of antigen exposure affect the speed of T-cell-mediated immunity. Naïve cells differentiating into memory CD4+ T cells can acquire effector function, secrete inflammatory cytokines, help B-cell responses and enhance CD8+ T-cell development. Different patterns of surface proteins can differentiate memory cells that reside in peripheral tissues or lymphoid organs, known as effector or central memory T cells. Both CD4+ and CD8+ T cells have a role in fighting *M. tuberculosis* infection; the organism resides in the phagosome of the macrophage and antigens are readily processed in association with MHC Class II. Mycobacteria-specific CD4+ T cells are T helper type 1 (Th1), potent IFN- γ producers and provide resistance against TB.^{3,4,8,11} Mice and humans with genetic deficiencies in IFN- γ or IL-12 receptor signalling pathways are known to be extremely susceptible to severe mycobacterial infections, illustrating their central role in containment of *M. tuberculosis* infection.⁹ Assays of type 1 immune responses, which involve IFN- γ production, may be useful as surrogate markers for protective immunity.¹⁸ It is, however, felt that long-term control of *M. tuberculosis* infection is associated not just with elevated Th1 responses but also with inhibition of the Th2 response.⁵

INTERFERON-GAMMA PRODUCTION

The lymphokine IFN- γ is thought to be a principal mediator of macrophage activation and resistance to intracellular pathogens.⁴ Extensive animal data indicate that the Th1 type of CD4+ T cells associated with high levels of IFN- γ secretion after antigenic stimulation is important in mycobacterial immunity.⁶ Although BCG vaccination effectively induces Th1-like immune responses, quantitation of IFN- γ responses after vaccination may not accurately reflect the degree of protection conferred.⁹

Whole-blood production of interferon-gamma

This assay has been described as 'the flawed current gold standard'.¹² Experimental models and case contact studies indicate that *M. tuberculosis*-stimulated whole-blood production of IFN- γ is the best available correlate of protection.¹² Measuring whole-blood production of IFN- γ after stimulation with *M. tuberculosis* antigens is a low-technology, inexpensive approach, and correlates well with IFN- γ production by peripheral blood mononuclear cells (PBMCs).¹²

The rationale for the choice of IFN- γ as a correlate includes the following:

- IFN- γ is essential for protective immunity against *M. tuberculosis* in genetically disrupted mice.¹² Mice homozygous for the targeted IFN- γ gene disruption ('gko mice') produce no IFN- γ , but are healthy in the absence of pathogens. However, *in vitro* activation of macrophages from these mice was greatly reduced and the ability of the mice to survive a BCG vaccine strain infection was impaired.⁴
- Patients with an IFN- γ -receptor abnormality are susceptible to atypical mycobacterial infection, dissemi-

nated BCG infection, and infection with *M. tuberculosis*.¹²

- Patients with moderate to advanced TB have depressed *M. tuberculosis*-stimulated whole-blood IFN- γ .¹²
- IFN- γ immunotherapy is clinically and bacteriologically beneficial in the treatment of multidrug-resistant TB.¹²
- Whole-blood production of IFN- γ is increased in individuals with *M. tuberculosis* infection but no disease – individuals relatively resistant to exogenous TB reinfection.¹²
- Whole-blood production of IFN- γ increases in subjects who have a TST conversion.¹²
- In pleural TB, IFN- γ concentrations and mRNA for IFN- γ in pleural fluid, as well as IFN- γ production by *M. tuberculosis*-stimulated pleural fluid cells, are all increased relative to similar measurements in blood.¹²
- Pleural TB has the potential to resolve without therapy. This suggests that the local immune response is protective in these patients.¹²

Factors against choosing whole-blood production of IFN- γ as a correlate of protection include the following:

- Ellner *et al.*¹² have demonstrated that IFN- γ levels vary in various stages of TB. In initial infection, before TST conversion, and in adults with moderate and especially advanced reactivated TB, levels tend to be low. In TST-positive children aged less than 5 years with progressive primary TB disease and in those who develop protective immunity, in TST-positive adults with latent TB infection and in adults with mild reactivated TB, levels tend to be high. Whole-blood production of IFN- γ is thus unable to distinguish infection from disease.¹²
- Some cases of progressive primary TB in children and some cases of minimal disease in adults self-cure without specific chemotherapy.
- IFN- γ fails to activate the killing of *M. tuberculosis* by human monocytes and in certain circumstances may promote intracellular replication.¹²

IFN- γ is essential for protection, but IFN- γ levels alone do not explain the immunity/susceptibility dichotomy.⁵ Reduced IFN- γ production by peripheral blood mononuclear cells has been found to be a marker of severe TB in both HIV-negative and HIV-infected patients with TB.⁶ Black *et al.*⁷ found that the magnitude of BCG-attributable increase in IFN- γ responsiveness to *M. tuberculosis* PPD, from before to 1 year post vaccination, correlated better with the known levels of protection induced by immunisation with BCG than did the absolute value of the IFN- γ or DTH response after vaccination.

Use of a monoclonal antibody specific for IFN- γ as a co-adjuvant in a subunit vaccine against TB resulted in an increased Th1 response characterised by enhanced IFN- γ production and cell proliferation, but did not result in enhanced protection against *M. tuberculosis* challenge. There is a need to identify further correlates of protection in addition to IFN- γ production to screen vaccines against TB infection.² In Malawian young adults, the 6-day IFN- γ response to PPD correlated well with Mantoux skin test induration. Discordant individuals were observed who were thought to possibly represent important subsets in terms of protective immunity and risk of clinical TB.²²

Hoft *et al.*⁹ evaluated the hypothesis that immune-mediated inhibition of mycobacterial growth would more directly correlate with protective TB immunity than other immunological responses. They found that BCG

significantly enhanced all antigen-specific responses, but that IFN- γ production did not correlate with mycobacterial inhibition.

Improved interferon-gamma assays

The IFN- γ assay may be refined by:

- Use of purified *M. tuberculosis* antigens that may be *M. tuberculosis*-specific and/or vaccine candidates
- Use of selected purified *M. tuberculosis* antigen cocktails
- Quantitation of IFN- γ production by mycobacteria-specific CD4 cells using flow cytometry performed on whole blood
- Assessing the ratio of IFN- γ responses induced by a purified antigen to the responses induced by crude antigenic preparations. Ideally, the purified antigen would be restricted in its species distribution to *M. tuberculosis* and would also be a vaccine candidate.
- Assessing the ratio of whole-blood IFN- γ production and tumour necrosis factor alpha (TNF- α). TNF- α levels are increased by inflammation during modulation of *M. tuberculosis* infection or disease. Preserved IFN- γ production in the presence of active inflammation may have a different significance to production in the absence of inflammation.
- Use of purified antigens and/or assessment of expression of IFN- γ by lymphocyte subpopulations by spot forming cells using the ELISpot test.

The following have also been suggested as possible immune correlates of protection against TB:

- Decreased intracellular mycobacterial survival in the blood¹⁰
- Inhibition of TNF- α ¹⁰
- Increased levels of Th1 cytokines⁵
- Decreased levels of IL-10⁵
- Selective increase of message for IL-4 delta2⁵
- Increase in CD4+ cells and CD4+/CD8+ ratio with Th1 type cytokine profile, e.g. selective concentration of IFN- γ , TNF- α , or IL-12.¹⁵
- Accelerated lymphoproliferative response to PPD¹⁵
- Increased *in vitro* IL-4 response together with apoptosis¹⁵
- Gamma-delta T cells may be capable of developing a memory immune-like phenotype, and therefore might be important targets for new vaccines⁶
- Assays that assess the killing of intracellular mycobacteria, using isolated monocytes or in a T-cell-dependent monocyte system^{9,12}
- Assessing CD4- and CD8-dependent cytotoxic effector mechanisms.¹²

RESEARCH NEEDS

Shinnick¹³ notes that research is needed to develop better tests that can distinguish potential vaccine failures from immunologically protected persons in a timely manner, as well as to distinguish the various outcomes of an infection. Research to identify stage-specific antigens or stage-specific immune responses holds some promise in this regard.

Other research needs include:

- Refinement of the IFN- γ assay¹²
- Longitudinal studies to examine BCG-induced responses in adolescents vaccinated at birth with BCG
- Immunological assays comparing the immune responses induced by live *M. tuberculosis* with *M.*

bovis BCG

- Studies of more complex assays that model *M. tuberculosis* killing¹²
- Studies of more complex assays that model cytotoxic T-lymphocyte activity¹²
- Household-contact studies allow the distinction between protection and certain forms of disease¹²
- Jason *et al.*¹⁷ suggest that it might be useful to develop an *in vitro* system to determine whether various antimycobacterial vaccines produce type 1 versus type 2 cytokine responses to mycobacterial antigens and examine these responses in relation to clinical efficacy.¹⁷

CONCLUSION

It has been noted that determination of true surrogate markers of protective TB immunity will require further knowledge of the specific human mechanisms of killing of intracellular *M. tuberculosis* and TB-specific regional lung immunity.⁹ Until a robust correlate of protection is available, a practical approach for detection of protective immunity induced by candidate TB vaccines might be to combine simple-to-perform and highly sensitive lymphoproliferation assays, with assays of mycobacterial killing, which potentially measure the most biologically relevant result.⁹ Recent advances in DNA chip technology should facilitate global analyses of the host responses to TB which will provide information on the correlates and surrogates of susceptibility and resistance as well as of protection.³

Dr Tony Hawkridge is supported by the Aeras Global Tuberculosis Vaccine Foundation.

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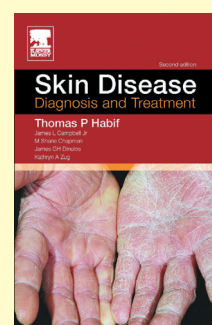
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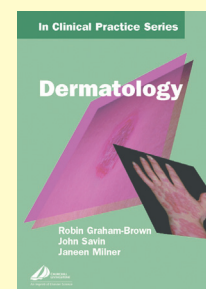
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