Boosting, BCG, and time of reading in tuberculin skin testing

Tuberculosis screening programmes of health-care workers are generally based on repeated tuberculin skin testing (TST), either at set intervals or on exposure to infection. Repeated testing can result in increased test reactions because it might evoke recall of delayed-type hypersensitivity to mycobacterial antigens that has waned.[1] Although this booster effect is greatest when the interval between the tests is 1 to 5 weeks, it can occur even after a year. Booster effects may cause false TST conversions.[1]

In many screening programmes, boosting is controlled for by two-step testing on entry into the programme. When the first step (T1) is negative, it is repeated after 1–2 weeks (T2). Since the likelihood of infection during this interval is very small, increases in reaction size are likely to be due to boosting, and the reaction to T2 can be taken as the baseline value for follow-up.[2] TST reactions are generally read after 2 to 4 days.

An important cause of boosting is past BCG vaccination. BCG affects the specificity of the TST. This effect wanes over time. At the first TST done 10 years after BCG vaccination, 15–25% of people will test positive, but another 15-5% will exhibit a booster response at T2.[3]

In a recent study done in the UK by Dave Singh and colleagues,[4] booster reactions in two-step testing seemed to vary greatly according to the time of reading. The investigators retested 26 BCG-vaccinated health-care workers after one week by the Mantoux method, using a PPD that is roughly bioequivalent to the international standard PPD-S. For each of the steps the results were read after 48 and 72 h. Singh and colleagues found a significant increase in induration between both tests (7·1 to 14·9 mm, $p<0·001$) when they were read at 48 h, but no change when read at 72 h (9·5 and 9·7 mm). With a cutoff of 15 mm for positive reactions, eight of 15 initially negative people became positive when interpretation was based on reactions read at 48 h, compared with six of 15 for readings at 72 h. However, for readings at 72 h, five of ten reactions that were positive after T1 became negative after T2, whereas no such reversions occurred when T2 was read at 48 h. The investigators conclude that, among BCG-vaccinated people retested after 1 week, the boosting effect falls off rapidly after 48 h and that the time of reading in two-step testing is therefore critical.

Do these findings merely reflect boosting, since the increase in induration between 48 and 72 h after T1 was more than 10 mm in four of the 26 participants? In a study done in the 1950s, there was only minimal variation in reactions at 48 and 72 h to a single TST.[5] Apart from an influence of BCG status, differences between readings may be due to differences in the PPDs used. The extent of non-specific reactogenicity can vary between bio-equivalent PPDs,[6] and perhaps in a time-dependent manner. However, the possibility of reading errors should not be discarded,[7] so Singh and colleagues' findings should be confirmed.

Little is known about the effect of time of reading on size of booster reactions. A study among BCG-vaccinated Danes showed that reactions to T2 read at 24 h and 72 h differed by less than 1 mm, but there were no data for 48 h and the interval between T1 and T2 was 3 months.[8] So perhaps the difference in reaction size observed by Singh and colleagues occurs only with short intervals between tests and would have disappeared by the time a person is tested because of a potentially infectious contact (eg, after 6 months). Interpretation of the two-step test would then be difficult, because it is unknown whether the apparent peak at 48 h is transient, or whether there is a transient suppression of recall of delayed-type hypersensitivity at 72 h. If the former is true, then a reading T2 at 48 h would reveal a booster effect that is irrelevant, because it would not be present on subsequent testing, and so would result in wrongful exclusions from further screening. If the latter is true, then by reading T2 at 72 h some booster reactions would be missed, thus resulting in false conversions later on. It is therefore important that these issues are clarified—for example, by studies in which BCG-vaccinated people with initially negative tests are retested at various time intervals, with the results being read at both 48 h and 72 h.

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1 Menzies D. Interpretation of repeated tuberculin tests. Boosting, conversion, and reversion. *Am J Respir Crit Care Med* 1999; **159:** 15–21.
2 Bass JA Jr, Serio RA. The use of repeat skin tests to eliminate the booster phenomenon in serial tuberculin testing. *Am Rev Respir Dis* 1981; **123:** 394–96.
3 Menzies D. What does tuberculin reactivity after Bacille Calmette-Guérin vaccination tell us? *Clin Infect Dis* 2000; **31** (suppl 3)**:** S71–74.
4 Singh D, Sutton C, Woodcock A. Repeat tuberculin testing in BCG-vaccinated subjects in the United Kingdom: the booster effect varies with the time of reading. *Am J Respir Crit Care Med* 2001; **164:** 962–64.
5 WHO Tuberculosis Research Office. Tuberculin reaction size on five consecutive days. *Bull World Health Organ* 1955; **12:** 189–96.
6 Comstock GW. A comparison of purified tuberculins in the South-eastern USA. *Bull World Health Organ* 1960; **23:** 683–88.
7 Fine PE, Bruce J, Ponnighaus JM, Nkhosa P, Harawa A, Vynnycky E. Tuberculin sensitivity: conversions and reversions in a rural African population. *Int J Tuberc Lung Dis* 1999; **3:** 962–75.
8 WHO Tuberculosis Research Office. Repeated tuberculin tests in the same site. *Bull World Health Organ* 1955; **12:** 197–209.

Gentamicin in pharmacogenetic approach to treatment of cystic fibrosis

Cystic fibrosis is due to mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is a member of the ATP-binding cassette (ABC) family of transmembrane transporter proteins. The mutations cause abnormal transport of both chloride and sodium across many types of epithelial tissues as well as disrupting other membrane-associated functions, including ion and pH regulation.[1–3] With the introduction of pancreatic-enzyme replacement therapy, the main clinical problem today is that of defective lung function and lung infections, which together account for over 90% of all deaths from cystic fibrosis.[4,5]

Although CFTR was among the first large human genes to be identified, cloned, and fully sequenced[6] and an early candidate for use in gene therapy, technical challenges remain evident even after more than a decade of effort with this form of therapy. Meanwhile, pharmacological approaches directed at specific biochemical defects underlying the disorder have continued to be pursued.

Almost a thousand individual CFTR mutations have been described, but they fall into five different functional classes (stop mutations, missense mutations, frameshifts, in-frame deletions, and splicing mutants) (figure), and only a few key mutations predominate in human beings. The mutation resulting in a deletion of the phenylalanine at position 508 of the protein (ΔF508), accounts for about two-thirds of all the cystic-fibrosis mutations that produce clinical effects.[4,5] This mutation is thought to cause

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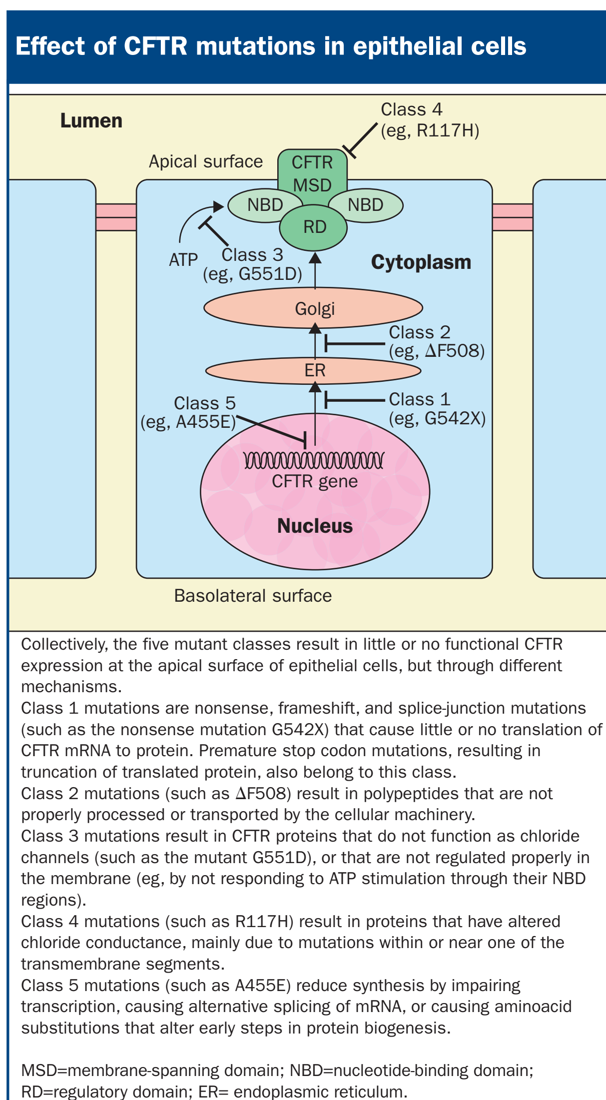

Collectively, the five mutant classes result in little or no functional CFTR expression at the apical surface of epithelial cells, but through different mechanisms.
Class 1 mutations are nonsense, frameshift, and splice-junction mutations (such as the nonsense mutation G542X) that cause little or no translation of CFTR mRNA to protein. Premature stop codon mutations, resulting in truncation of translated protein, also belong to this class.
Class 2 mutations (such as ΔF508) result in polypeptides that are not properly processed or transported by the cellular machinery.
Class 3 mutations result in CFTR proteins that do not function as chloride channels (such as the mutant G551D), or that are not regulated properly in the membrane (eg, by not responding to ATP stimulation through their NBD regions).
Class 4 mutations (such as R117H) result in proteins that have altered chloride conductance, mainly due to mutations within or near one of the transmembrane segments.
Class 5 mutations (such as A455E) reduce synthesis by impairing transcription, causing alternative splicing of mRNA, or causing aminoacid substitutions that alter early steps in protein biogenesis.

MSD=membrane-spanning domain; NBD=nucleotide-binding domain; RD=regulatory domain; ER= endoplasmic reticulum.

defective folding of CFTR in the endoplasmic reticulum and thus to prevent it from moving efficiently through the Golgi apparatus;[5,7,8] the result is that expression of CFTR is dampened at the cell membrane. Even a modest increase in functional CFTR expression, to only a fraction of normal levels, is thought to be sufficient to help correct the cystic-fibrosis phenotype, hence the interest in overcoming this trafficking defect. Recently, several pharmacological agents, among them 4-phenylbutyrate and the anthracycline doxorubicin, have been reported to specifically increase the folding, trafficking, and functional cell-surface expression of ΔF508 CFTR.[9–12]

Another type of mutation that is prominent among patients with cystic fibrosis is stop mutations (ie, those that prematurely terminate translation of the CFTR mRNA into functional protein). They affect about 10% of these patients and are especially common among certain groups. For example, they are found in up to 85% of patients of Ashkenazi Jewish descent.

Bedwell and colleagues had reported that aminoglycoside antibiotics could overcome the effect of stop mutations within the CFTR gene in respiratory epithelial cells cultured from patients harbouring these mutations, to result in read-through of the CFTR mRNA.[13,14] Others had shown in a mouse model that aminoglycosides could also increase expresssion of full-length mRNA from a dystrophin gene that contained stop codon mutations.[15] Now J P Clancy and colleagues[16] report that gentamicin given to five patients for a week increased functional CFTR expression in the airway. These increases occurred in patients harbouring stop mutations, but not in those with other CFTR mutations.

Clancy and colleagues obtained further evidence that gentamicin boosts functional CFTR expression largely by increasing read-through of the CFTR mRNA in the airway epithelial cells by measuring membrane potential difference (PD) in the nasal epithelium of these patients. Nasal PD was higher among those with than those without stop mutations (and four of five patients with stop mutations had a hyperpolarised response of greater than −5 mV). Ex-vivo examination of nasal epithelial cells from patients who were treated with gentamicin showed a threefold increase in apical surface expression of CFTR (as measured by confocal imaging) and a significant increase in halide efflux (as measured by a fluorescence assay). In-vivo tests of sweat glands and lung spirometry did not reveal a significant difference between the two cystic-fibrosis groups. Clancy and colleagues' reasonable explanation is that spirometry readings would not be expected to change with such a short course of treatment, and that the sweat test, although qualitatively diagnostic for cystic fibrosis, is not a good quantitative indicator of clinically important aspects of the disease.

The findings hold promise for the development of effective treatment protocols. Gentamicin should be viewed as a lead compound, and the future goal will be to identify and/or synthesise analogues that will maximise their clinical and biochemical effects on CFTR while minimising gentamicin-mediated toxicity or side-effects. Reduction of adverse effects will be important since these drugs would probably need to be given for life. This general pharmacogenetic approach[17] of developing agents to override the effect of stop mutations might perhaps be extended beyond cystic fibrosis to other diseases caused by such mutations, which includes most of the common autosomal recessively inherited disorders.

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1 Robinson LJ, Roepe PD. Effects of membrane potential versus pH_i on the cellular retention of doxorubicin analyzed via a comparison between cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance (MDR) transfectants. *Biochem Pharmacol* 1996; **52:** 1081–95.
3 Stutts MJ, Canessa CM, Olsen JC, et al. CFTR as a cAMP-dependent regulator of sodium channels. *Science* 1995; **269:** 847–50.
3 Al-Awqati Q. Regulation of ion channels by ABC transporters that secrete ATP. *Science* 1995; **269:** 805–06.
4 Zielenski J, Tsui L-C. Cystic fibrosis: genotypic and phenotypic variations. *Annu Rev Genet* 1995; **29:** 777–807.
5 Davis PB, Drumm M, Konstan MW. Cystic fibrosis. *Am J Respir Crit Care Med* 1996; **154:** 1229–56
6 Zielenski J, Rozmahel R, Bozon D, et al. Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* 1991; **10:** 214–28.
7 Thomas PJ, Qu B-H, Pedersen PL. Defective protein folding as a basis of human disease. *TIBS* 1995; **20:** 456–59.
8 Sheppard DN, Ostedgaard LS. Understanding how cystic fibrosis mutations cause a loss of Cl^- channel function. *Mol Med Today* 1996; **2:** 290–97.
9 Guay-Broder C, Jacobson KA, Barnoy S, et al. A_1 receptor antagonist 8–cyclopentyl-1,3–dipropylxanthine selectively activates chloride efflux from human epithelial and mouse fibroblast cell lines expressing the cystic fibrosis transmembrane regulator ΔF508 mutation. *Biochemistry* 1995; **34:** 9079–87.

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10 Rubenstein RC, Egan ME, Zeitlin PL. In vitro pharmacologic restoration of CFTR-mediated chloride transport with sodium 4–phenylbutyrate in cystic fibrosis epithelial cells containing ΔF508–CFTR. *J Clin Invest* 1997; **100:** 2457–65.

11 Zeitlin PL. Novel pharmacologic therapies for cystic fibrosis. *J Clin Invest* 1999; **103:** 447–52.

12 Maitra R, Shaw CM, Stanton BA, Hamilton JW. Increased functional cell surface expression of CFTR and ΔF508–CFTR by the anthracycline doxorubicin. *Am J Physiol Cell Physiol* 2001; **280:** C1031–C1037.

13 Howard M, Frizzell RA, Bedwell DM. Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. *Nat Med* 1996; **2:** 467–69.

14 Bedwell DM, Kaenjak A, Benos DJ, et al. Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. *Nat Med* 1997; **3:** 1280–81.

15 Barton-Davis ER, Cordier L, Shorurma DI, Leland SE, Sweeney HL. Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mds* mice. *J Clin Invest* 1999; **104:** 375–81.

16 Clancy JP, Bebok Z, Ruiz F, et al. Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2001; **163:** 1683–92.

17 Wieczorek SJ, Tsongalis GJ. Pharmacogenomics: will it change the field of medicine? *Clin Chim Acta* 2001; **308:** 1–8.

Trends in reporting of SNP associations

Single nucleotide polymorphisms (SNPs) are genetic markers that are usually diallelic. In recent years they have gained in popularity because of their high prevalence in the genome and their amenability to automated analyses. Before 1996 linkage analysis had been the mainstay of early-stage discovery of genes that predispose to disease, but in 1996 Risch and Merikangas highlighted the potential of an older technique, association analysis, for detecting alleles that produce modest phenotypic effect.[1] Now, 5 years later, R U Uhl and colleagues[2] have completed a genome-wide SNP association study. They genotyped more than 1000 people for almost 1500 SNPs, to identify genetic loci associated with vulnerability to polysubstance abuse.

This case-study sets a precedent for the reporting of results of widescale association testing. In its most general form, association analysis consists of a comparison of allele frequency between cases and controls and, as with other statistical techniques, a correction for multiple testing is necessary when several SNPs (or multiple traits) are analysed.

Risch and Merikangas[1] had noted that under some simplifying assumptions, a significance threshold of $p=5\times10^{-8}$ would produce a genome-wide false-positive rate of 5%. However, this guideline was based on a Bonferroni correction which, given the correlations between adjacent SNPs, is conservative and potentially damaging to statistical power.

There are methods less stringent than the Bonferroni correction and more suitable for correlated data. A good example is the Ryan-Holm step-down procedure, an easy-to-implement approach championed by Ludbrook[3] and based on earlier work by Ryan[4] and Holm.[5] Unfortunately, such methods have yet to enter common usage. In practice, particularly for candidate-gene studies, many investigators have chosen instead to cite unadjusted p values, while providing the reader with sufficient detail to make his or her own adjustment.

In a departure from tradition, Uhl and colleagues made no mention of classic p values for their genome scan.[2] Instead they reported a measure of association, similar to an odds ratio, which they termed the abuser/control ratio, without making any assumptions about its distribution. As with other pool-based association tests, their analysis makes use of some untested assumptions about Hardy-Weinberg equilibrium and lack of population stratification. Two features of their approach signal a growing trend. First, they examined two ethnically independent study populations, to enable immediate confirmatory analysis and, second, they used Monte Carlo simulation (a process of repeatedly selecting SNPs at random from the total set) and empirical reasoning to assess findings.

Uhl and colleagues make the efficient and reasonable assumption that the abuser/control ratios derived from almost 1500 SNPs provide an empirical distribution of the ratios under the null hypothesis of no association. They then selected the 5% tails as initial positives. Repetition of the experiment in an independent sample enabled the identification of loci that were “reproducibly positive”. Monte Carlo simulations then allowed the empirical estimation of the probability of deriving such concordant results by chance.

This approach is strong and well accepted but computationally intensive. Other options are available and should be explored. Vieland and Hodge,[6] for example, forwarded a powerful argument for the reporting of likelihood ratios as measures of statistical evidence. Likelihood ratios, unlike classic p values, do not rely on assumptions about distributions but are based solely on observed data. Furthermore they are not subject to varying interpretation; p values, on the other hand, may become non-significant as additional tests are done.

The decreasing emphasis on classic p values and the increasing scrutiny of replication studies means that it is more important than ever for investigators to provide a clear and cohesive description of what exactly they have done. Reports should contain, at a minimum, sample sizes, ascertainment methods, matching schemes, and summary statistics for age, sex, ethnicity, and any obvious covariates.

After Horikawa and colleagues[7] reported the association between SNPs in calpain 10 (CAPN10) and type 2 diabetes, there followed a flurry of validation studies, with numbers providing a measure of confirmation about equal to those providing no confirmation.[8] The fact remains that conclusive confirmation can be obtained only from biological characterisation of the gene and its protein by, for example, expression studies. However, a balanced evaluation of whether these costly steps should be undertaken depends less on p values and more on the detailed and complete reporting of the methods that preceded them.

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1 Risch N, Merikangas K. The future of genetic studies of complex human diseases. *Science* 1996; **273:** 1516–17.

2 Uhl RU, Liu QR, Walther D, Hess J, Naiman D. Polysubstance abuse-vulnerability genes: genome scans for association using 1004 subjects and 1494 single nucleotide polymorphisms. *Am J Hum Genet* 2001; **69:** 1290–300.

3 Ludbrook J. Multiple comparison procedures updated. *Clin Exp Pharmacol Physiol* 1998; **25:** 1032–37.

4 Ryan TA. Significance tests for proportions, variances, and other statistics. *Psychol Bull* 1960; **57:** 318–28.

5 Holm S. A simple sequentially rejective multiple test procedure. *Scand J Statist* 1979; **6:** 65–70.

6 Vieland VJ, Hodge SE. Book review of Statistical Evidence: likelihood paradigm by Richard Royall (London: Chapman and Hall, 1997). *Am J Hum Genet* 1998; **63:** 283–89.

7 Horikawa Y, Oda N, Cox NJ, et al. Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nat Genet* 2000; **26:** 163–75.

8 Tsai H-J, Sun G, Weeks DE, et al. Type 2 diabetes and three calpain-10 gene polymorphisms in Samoans: no evidence of association. *Am J Hum Genet* 2001; **69:** 1236–44.

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