Identifying genetic susceptibility factors for tuberculosis in Africans: a combined approach using a candidate gene study and a genome-wide screen*

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ABSTRACT

There is convincing evidence that host genes affect the outcome of infection in human tuberculosis. Two complementary strategies were used to identify the genes involved. A linkage-based genome-wide screen was carried out to locate the positions of genes exerting a major population-wide effect on tuberculosis susceptibility. A candidate-gene-based case–control study was used to examine the effects of genes that may exert a more moderate effect on risk of clinical tuberculosis. The genome screen was conducted in two stages. In the first stage 299 microsatellite markers, spanning all 23 chromosomes, were typed in 92 independent sib-pairs, and seven regions showed some evidence of co-segregation with the disease. These seven regions were examined in a second set of 81 sib-pairs, and markers on chromosomes 15q and Xq showed evidence of linkage to tuberculosis. An X chromosome susceptibility gene may contribute to the excess of males with tuberculosis observed in many populations. The candidate gene approach compared the frequency of polymorphisms in several genes in over 400 subjects with smear-positive pulmonary tuberculosis and 400 ethnically matched healthy controls. Polymorphisms in genes encoding natural-resistance-associated macrophage protein, vitamin D receptor and mannose-binding lectin were associated with tuberculosis. These results suggest that many genes may be involved in determining host susceptibility to tuberculosis, and highlight the importance of using several different study methods to locate them.

INTRODUCTION

It is estimated that one-third of the World’s population is infected with *Mycobacterium tuberculosis*, but that only one in ten of those infected ever develop clinical disease [1,2]. There are many well known risk factors for tuberculosis, including HIV infection, advanced age, malnutrition, alcohol abuse, diabetes and corticosteroids. However, many patients do not have any obvious underlying risk factor, and there is now convincing evidence that individual variability in tuberculosis susceptibility is partly determined by host genes [3]. Racial differences in resistance to tuberculosis may be determined by history of exposure among previous generations [4,5], and concordance for tuberculosis is higher among identical than non-identical twins [6,7].

* This paper was presented at the Glaxo/MRS Young Investigator session at the MRS Meeting, Royal College of Physicians, London, on 19 May 1999.

Key words: genetic susceptibility, genome screen, mannose-binding lectin, Nramp, tuberculosis, vitamin D receptor.
Abbreviations: MBL, mannose-binding lectin; NRAMP/Nramp, natural-resistance-associated macrophage protein; VDR, vitamin D receptor.
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M. tuberculosis is a facultative intracellular pathogen which utilizes the macrophage as its host cell. Resistance to M. tuberculosis involves a complex interaction between the bacteria and the host immune system. Several mechanisms have been proposed to explain how the macrophage combats mycobacterial resistance. These include the generation of reactive oxygen and reactive nitrogen intermediates, acidification of phagosomes, phagosome–lysosome fusion and the limitation of intraphagosomal iron [8]. It is uncertain how M. tuberculosis evades these mechanisms. Hypotheses include invading macrophages via C3b receptors to evade the production of reactive oxygen intermediates [9], production of ammonia to resist phagosome acidification [10], and inhibition of phagosome–lysosome fusion [11]. Cytokines are believed to play a key role in mycobacterial resistance. Macrophages are activated by interferon γ, tumour necrosis factor α, vitamin D and interleukin 6 [11]. These cytokines may play an important role in resistance to tuberculosis, although they may also be responsible for much of the tissue destruction that occurs in this disease [12].

Two approaches were used to investigate the role of host genes in susceptibility to tuberculosis in Africans. A linkage study was used to screen the whole human genome for regions containing potential major tuberculosis-susceptibility loci. This approach is systematic and comprehensive, but has a relatively low power of detection and would fail to identify any genes exerting only a moderate effect on disease susceptibility. It has been calculated that if a susceptibility allele with a population frequency of 0.5 exerts a 2-fold increased risk of disease, 2498 sib-pairs would be required to provide 80% power to detect the effect by linkage analysis [13]. An association (case–control) study would only require 340 cases to provide the same power [13], and can therefore detect smaller population-wide genetic effects. Association studies cannot currently be used to perform genome-wide screens, as at least 3000 markers would need to be typed. Our case–control study was therefore restricted to candidate genes.

The study described in this review was approved by the joint ethics committee of the Gambian Government and the Medical Research Council. Verbal consent was obtained from all study subjects.

RESULTS OF THE GENOME-WIDE SCREEN

The genome screen was carried out using sib-pair families. These are families with two or more full siblings affected by the same disease (tuberculosis in this case). A family containing two affected siblings provides one sib-pair, one containing three affected siblings provides two fully independent sib-pairs, and if there are four affected siblings this is equivalent to three independent sib-pairs, etc. If a genetic marker locus is linked to a major disease-susceptibility gene, then the siblings will share the parental alleles that they have inherited at that locus more often than would be expected by chance. This non-parametric system of analysis is not dependent on the mode of inheritance, and a major disease-susceptibility locus should be identifiable regardless of whether it is X-linked or autosomal, dominant or recessive.

The genome screen was carried out in two stages, with approximately half of the sib-pairs in each stage. This is more efficient than carrying out the whole genome-wide screen on all of the families. In the first stage, 299 microsatellite markers covering all 23 chromosomes were typed in 67 Gambian families including 73 fully independent sib-pairs, and in 16 KwaZulu–Natal families including 19 independent sib-pairs. A total of seven regions, on chromosomes 3, 5, 6, 8, 9, 15 and X, showed nominal evidence for linkage to tuberculosis (R. Bellamy, N. Beyers, C. Ruwende, H. C. Whittle, E. Hoal van Helden, T. Corrah, R. Wilkinson, K. P. W. J. McAdam, W. Amos, P. van Helden and A. V. S. Hill, unpublished work). These results could have been due to genuine linkage or simply to chance, because of the large number of loci typed, and therefore needed replication in a second series of families. No region showed evidence of strong linkage, indicating that in these populations genetic susceptibility to tuberculosis is not determined by a single-gene effect.

In the second round of the screen, 22 markers from the seven regions identified in the first stage of the screen were typed in 12 Gambian sib-pair families and in 41 families including 69 independent sib-pairs from the Western Cape, South Africa. Two regions, on chromosome Xq26 and the pericentricromeric region of chromosome 15q, showed evidence of linkage to a putative tuberculosis-susceptibility gene (R. Bellamy, N. Beyers, C. Ruwende, H. C. Whittle, E. Hoal van Helden, T. Corrah, R. Wilkinson, K. P. W. J. McAdam, W. Amos, P. van Helden and A. V. S. Hill, unpublished work). Several candidate genes lie in the region of interest on Xq26, and the CD40 ligand in particular is worthy of further investigation. It is of note that there are approximately twice as many male as female patients with tuberculosis in The Gambia and South Africa (R. Bellamy, unpublished work; [14]). This significant male excess has been observed in many different ethnic groups [15–17], and our results suggest that it may be due to an X-linked tuberculosis-susceptibility gene.

CANDIDATE GENE STUDY

Subjects for the case–control study were all recruited from The Gambia. The relative ethnic homogeneity of the population [18] make this an ideal population for case–control studies. All patients were HIV-negative.
NRAMP
Among inbred strains of mice, natural resistance to infection with several antigenically unrelated intracellular pathogens is controlled by a single dominant gene on mouse chromosome 1, designated Bcg (also known as Lsh/Ity) [23,24]. Two distinct non-overlapping phenotypes are recognized, Bcg<sup>+</sup> and Bcg<sup>−</sup>, which are respectively susceptible and resistant to the early stage of infection with <i>Mycobacterium bovis</i> (BCG), <i>M. intracellulare</i>, <i>M. lepraemurium</i>, <i>Leishmania donovani</i> and <i>Salmonella typhimurium</i> [23–28]. A candidate gene for Bcg has been isolated by positional cloning and designated <i>Nramp1</i> (murine natural-resistance-associated macrophage protein) [29]. <i>Nramp1</i> and Bcg/Lsh/Ity were proven to be identical by the production of a gene-disrupted <i>Nramp1</i> knockout mouse [30], and an <i>Nramp1</i> transgenic mouse which restored the wild-type resistant phenotype [31]. It has been shown that <i>Nramp1</i> is not the sole major gene determining resistance to infection with virulent <i>M. tuberculosis</i> [32] in mice, but it may have a more complex role inmurine <i>M. tuberculosis</i> immunity in conjunction with other host genes.

The human homologue of the <i>Nramp1</i> gene, designated <i>NRAMP1</i>, has been cloned and mapped to chromosome 2q35 [33,34]. Several polymorphisms have been described in <i>NRAMP1</i>, and it has been suggested that they could influence <i>NRAMP1</i> function [34,35]. Weak evidence of linkage has been found between <i>NRAMP1</i> markers and susceptibility to leprosy in 20 families from Vietnam (P < 0.02), although this was not confirmed in seven families from French Polynesia [36,37]. Weak evidence of linkage to the <i>NRAMP1</i> locus was also found in our own study of tuberculosis in families (P = 0.06) (R. Bellamy, unpublished work) and in a study of 98 Brazilian families (P = 0.025) [38]. The absence of strong evidence of linkage in these studies indicates that <i>NRAMP1</i> is not the sole determinant of host genetic susceptibility to mycobacterial infections in humans.

Case–control studies have much greater power than linkage studies for investigating the complex role of candidate genes in multifactorial diseases. Six <i>NRAMP1</i> polymorphisms were typed in our large Gambian case–control study. Four polymorphisms were strongly associated with tuberculosis in this population (overall P = 0.000008; one gene variant was not associated and the other was too rare to evaluate) [20]. Individuals who possessed two variant alleles were 4-fold over-represented among the tuberculosis cases compared with the healthy controls (Table 1; [20]). This result demonstrates that, although <i>NRAMP1</i> is not the sole determinant of host tuberculosis susceptibility, it is an important mycobacteria-susceptibility gene in humans as well as in mice. The functions of Nrap1/NRAMP1 remain unknown, although it is believed that they may restrict the availability of iron to intraphagosomal mycobacteria [39].

VDR
Genetic variation in bone mineral density has been shown to be associated with polymorphisms in the <i>VDR</i> gene in many, although not all, populations studied [40]. Epidemiological evidence suggests a link between vitamin D deficiency and susceptibility to tuberculosis [41,42], and vitamin D has been shown to have beneficial effects in cutaneous tuberculosis [43]. Vitamin D is an important immunoregulatory hormone [44]. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-dihydroxycholecalciferol), activates monocytes, stimulates cell-mediated immunity and suppresses lymphocyte proliferation, immunoglobulin production and cytokine synthesis [44]. <i>In vitro</i> studies suggest that vitamin D metabolites can enhance the ability of human monocytes...
controls (represented in the tuberculosis cases compared with the
which has been designated tt, was significantly under-
typed in our Gambian tuberculosis case–control study.
may influence susceptibility to infectious diseases.
will be required to investigate how
and the lepromatous form of leprosy [47]. Further studies
to develop persistence of hepatitis B virus infection [21]
with this genotype have also been found to be less likely
zygotes may be resistant to clinical tuberculosis. Subjects
confidence interval 0.31–0.88), suggesting that tt homo-
with those of genotypes TT
GBans,
population possessing two mutant alleles [49]. Among
healthy British population, with around 4.6% of the
and have a combined frequency of 22.5% among a
However,
MBL, and are predisposed to recurrent childhood
infections and possibly to infections in adult life [48].
not be found to be less likely
to develop persistence of hepatitis B virus infection [21]
and the lepromatous form of leprosy [47]. Further studies
will be required to investigate how VDR polymorphism
may influence susceptibility to infectious diseases.

MBL
MBL is a calcium-dependent serum lectin. It interacts
with the immune system by acting as an opsonin to
promote phagocytosis and by activating the complement
cascade. Three co-dominant single-base substitutions in
codons 52, 54 and 57 of the MBL gene result in reduced
serum MBL concentrations. Individuals with two variant
alleles have extremely low or undetectable levels of serum
MBL, and are predisposed to childhood infections and possibly to infections in adult life [48].
However, MBL variant alleles are extremely common
and have a combined frequency of 22.5% among a
healthy British population, with around 4.6% of the
population possessing two mutant alleles [49]. Among
Gambians, MBL variant alleles have a combined fre-
cuency of 31%, and around 10% of the population carry
two mutant alleles [22]. This is surprisingly high, because
infections are a frequent cause of premature death
throughout Africa and we might therefore expect strong
selection against MBL variants.
It has been suggested that heterozygote advantage may
maintain MBL variant alleles at high frequency by
conferring resistance to mycobacterial diseases [50]. In
our case–control study in The Gambia it was found that
the frequency of the common African variant MBL allele
(codon 57) was lower among tuberculosis cases than
controls (P = 0.037) [22]. This result will require confir-
mation in other populations due to its borderline
statistical significance. However, it would be fascinating
if MBL deficiency, described as the world’s commonest
immune deficiency, has been selected for by conferring
resistance to tuberculosis.

CONCLUSIONS
Recent work on subjects with extreme susceptibility to
atypical mycobacteria and other intracellular pathogens
has identified rare immune deficiencies due to abnor-
malities of the genes encoding the interferon γ receptor,
interleukin 12 and the interleukin 12 receptor [51–54].
These studies provide important candidate genes for
investigation in large population-based studies of com-
mon, related infections. It is likely that a large number of
host genes are involved in susceptibility to mycobacteria
and other infectious diseases. A variety of study designs,
including family-based linkage studies, large-scale popu-
lation case–control studies, investigation of unexplained
immune deficiencies and comparisons with animal
models of disease, will be required to identify further
host genes and investigate their interactions. Identifying
the critical determinants of host genetic susceptibility to
specific pathogens will further our understanding of the
pathogenesis of these diseases, and hopefully suggest new
treatment strategies.

ACKNOWLEDGMENTS
I am grateful to Professor A. V. S. Hill for helpful
discussions, and to the Wellcome Trust for funding.

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