Association of Vitamin D Receptor Genotype with Leprosy Type


Host genetic factors including major histocompatibility complex (MHC) polymorphisms influence both susceptibility to leprosy per se and also to leprosy type. Non-MHC genes may play an important role, but such genes remain undefined. The influence of two non-MHC candidate genes was assessed in a case-control study of Bengali leprosy patients from Calcutta. Recent studies have implicated variation in the vitamin D receptor (VDR) gene in susceptibility to several diseases, including osteoporosis and pulmonary tuberculosis. In this population, homozygotes for the alternate alleles of the VDR polymorphism are associated, respectively, with lepromatous and tuberculoid leprosy. The NRAMP1 (natural resistance associated macrophage protein 1) gene may influence human mycobacterial disease susceptibility based on studies with the murine homologue Nramp1. However, no significant association was found between NRAMP1 and leprosy susceptibility. This study suggests that the VDR polymorphism may influence susceptibility to some diseases by affecting the type and the strength of the host immune response.

Twin studies, interpopulation comparisons, and analysis of familial clustering have cumulatively provided strong evidence of a significant host genetic component in susceptibility to leprosy and, less clearly, to leprosy type [1–4]. The two polar types of leprosy differ from each other in that tuberculoid leprosy presents with few bacilli in macrophages and a strong cell-mediated response, and lepromatous leprosy presents with numerous bacilli and a weak cellular response. Studies of major histocompatibility complex (MHC) polymorphism have found evidence of linkage and association for both leprosy per se and for tuberculoid leprosy [3, 4]. In Asian populations, both types of leprosy are associated with HLA-DR2 [5]. However, these MHC effects appear insufficient to explain the whole host genetic component to susceptibility, and recent attention has focused on non-MHC genes, such as the NRAMP1 gene encoded on chromosome 2 and the vitamin D receptor on chromosome 12.

1,25 dihydroxyvitamin D$_3$ (1,25D3), the active form of vitamin D, not only regulates calcium and bone metabolism but also has an immunoregulatory role mediated through binding to the vitamin D receptor (VDR) in monocytes, macrophages, and activated lymphocytes [6]. A TaqI restriction fragment length polymorphism defines a single base change, C to T, in codon 352 at the 3’ end of the VDR gene [7, 8]. This site is in very strong linkage disequilibrium with a neighboring BsmI site polymorphism and in less strong linkage disequilibrium with a cluster of other sequence changes at the 3’ end of this gene. The less common allele of the TaqI site, designated “t,” has been associated with higher levels of mRNA expression in transient transfection assays [8]. The tt genotype (or tightly linked BB BsmI genotype) has been associated with lower bone mineral density, resistance to primary and secondary hyperparathyroidism, and resistance to prostatic cancer [7, 9–11]. Recently, this tt genotype has also been associated with enhanced clearance of hepatitis B virus infection and resistance to pulmonary tuberculosis in West Africans (unpublished data), providing further evidence of the functional relevance of this region and suggesting its possible relevance to leprosy susceptibility.

A single amino acid change in the NRAMP1 murine homologue, Nramp1, has been found to be causally associated with susceptibility to several intracellular pathogens, including bacille Calmette-Guérin, Mycobacterium leprae, and species of Salmonella and Leishmania [12], leading to the proposal [13] that NRAMP1 may be a major gene for susceptibility to human mycobacterial infection and disease. Several polymorphisms have been identified in NRAMP1 [13], but it is unclear which, if any, of these is of functional importance. In the 5’ flanking region of the NRAMP1 promoter is a putative Z-DNA-forming dinucleotide repeat which is polymorphic [14]. Four alleles have been described in this microsatellite, and it has been proposed that these may be associated with variable NRAMP1 gene expression [14]. Another polymorphic micro-
satellite has been described in exon 2 of NRAMP1 [15]. White et al. [15] identified a \(3 \times 9\) nucleotide repeat in exon 2 encoding the amino acid sequence SPTSPTSPPG. In a study of 69 unrelated Brazilians, the frequency of the rare allele (2 × 9, i.e., a 9-nt deletion) was found to be 0.02 [15]. Also, described are polymorphisms in the fourth intron (G/C) and a TGTG (4-base) deletion (1729 + 55 del 4) in the 3’ untranslated region [13]. A linkage study carried out in French Polynesian families [16] found no evidence that NRAMP1 was linked to leprosy susceptibility, whereas another small family study by Abel et al. [17] found significant nonrandom segregation of NRAMP1 haplotypes to affected siblings. A large case-control study of tuberculosis and NRAMP1 [18] showed that particular genetic variants in the NRAMP1 gene were associated with susceptibility to tuberculosis in West Africa.

We report herein an analysis of the polymorphisms in the NRAMP1 gene and the TaqI restriction fragment length polymorphism in codon 352 of the VDR gene in leprosy and control samples collected from Calcutta, India.

Materials and Methods

Subjects

Unrelated leprosy patients affected by the two polar types of disease were recruited at the School of Tropical Medicine, Calcutta, West Bengal, India. Diagnosis was based on the appearance and distribution of skin lesions, anesthesia, conditions of peripheral nerves, and acid-fast bacilli in slit-skin smear examination. Lepromatous leprosy required the presence of diffuse and a large number of skin lesions with numerous acid-fast bacilli in the slit-skin smears taken from at least three parts of the body, including one from an ear lobe. Tuberculoid leprosy was defined by the presence of asymmetrical well-defined lesions with a dry surface and the absence of detectable acid-fast bacilli. Borderline leprosy patients were not recruited for this study. The control samples were from professional blood donors visiting the Swasti Blood Clinic, Calcutta. Controls included for this study were unrelated and seronegative for antibodies to human immunodeficiency virus, Treponema pallidum, and hepatitis B surface antigen. Both patients and control groups included Hindus, Muslims, and Christians, and the Hindus included Brahmins, Vaidyas, Kaithhas, and Shudras. Patients and controls were thus prospectively matched for ethnic group.

DNA was extracted using a saturated sodium chloride solution [19], and analysis was performed by amplification of the relevant segment of the genes of interest.

Allele-Specific Oligonucleotide Probing

Denatured polymerase chain reaction (PCR) product was transferred to positively charged nylon membranes and probed with a digoxigenin-labeled probe using the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany). VDR The primers 5’-CAGAGCATGAGACGGGACAG-3’ and 5’-GGTGGCGCAGCAGGGATGTACG-3’ yielding a product of 340 bp were used. The PCR cycle conditions were 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s (35 cycles), using 2 mM MgCl₂, 0.2 mM dNTPs, 0.009 mM of each primer, 100 ng of DNA, and 1 U of Taq polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, CT) in a 25-μL reaction. Sequence-specific oligonucleotides for T (5’-GGCGTATGAGCCCATC-3’) and t (5’-GGCGTGA-TCAGGCCCATC-3’) alleles were used.

TaqI deletion. A product of 244 bp was obtained using the primers 5’-TCTCTGGCTGAAGGCTTCC-3’ and 5’-TGTGCTATCCG-TTGAAGCCTC-3’. The PCR cycle conditions were 94°C for 14 min, followed by 94°C for 10 s, 58°C for 20 s, and 72°C for 30 s (35 cycles), using 3 mM MgCl₂, 0.32 mM dNTPs, 0.1 μM each primer, 100 ng of DNA, and 1 U of Taq polymerase (AmpliTaq Gold; Perkin-Elmer) in a 25-μL reaction. Sequence-specific oligonucleotides for G (5’-TTGGGGGGCTTGAG-3’) and C (5’-TTGG-GGGCCCCGTGAC-3’) alleles were used.

Microsatellite Typing

Fragment size analysis was carried out using ABI 373 sequencing machines and Genescan and Genotyper software (Perkin-Elmer).

NRAMP1 promoter polymorphism. PCR amplification with the primer 5’-ACTCGCATAGGGCCAACG-3’ and TET-labeled reverse primer 5’-TCTCTGGCTCTCCCAAATGAC-3’ was done. The PCR cycle conditions were 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s (30 cycles), using 100 ng of DNA, 2.5 mM MgCl₂, 0.2 mM dNTPs, 2.5 ng/mL each primer, and 1 U of Taq polymerase (AmpliTaq Gold) in a 15-μL reaction mixture. Alleles 1, 2, and 3 were of 202, 200, and 198 bp, respectively.

| Table 1. Leprosy and vitamin D receptor (VDR) genotype. |
|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **Genotype**    | **TT**          | **Tt**          | **tt**          | **Total**       |
| Controls        | 66 (39.8)       | 87 (52.4)       | 13 (7.8)        | 166             |
| Tuberculoid     | 40 (37.4)       | 44 (41.1)       | 23 (21.5)       | 107             |
| Lepromatous     | 65 (52.4)       | 46 (37.1)       | 13 (10.5)       | 124             |

NOTE. Data are no. (%). Overall distribution of VDR genotypes is significantly different between lepromatous and tuberculoid leprosy (3 \(\chi^2\) = 7.6, \(P = 0.02\); corrected \(\chi^2 = 7.3\), \(P = 0.03\)). Difference between tuberculosis cases and controls (3 \(\chi^2\) = 11.0, \(P = 0.004\); corrected \(\chi^2 = 10.8\), \(P = 0.005\)) and between lepromatous leprosy cases and controls (3 \(\chi^2\) = 6.7, \(P = 0.04\); corrected \(\chi^2 = 6.6\), \(P = 0.04\)) were of marginal and no significance, respectively. \(\chi^2\) genotype is increased in tuberculoid leprosy cases vs. controls (\(\chi^2 = 10.6\), \(P = 0.001\), odds ratio [OR] = 3.32, 95% confidence interval [CI] = 1.47–7.13; corrected \(\chi^2 = 10.3\), \(P = 0.001\)). TT genotype is increased in lepromatous cases vs. controls (\(\chi^2 = 4.6\), \(P = 0.03\), odds ratio = 1.67 [95% CI: 1.02–2.75]; corrected \(\chi^2 = 4.4\), \(P = 0.04\)). Tt heterozygotes are reduced in frequency in leprosy patients as a whole (both types) vs. controls (\(\chi^2 = 7.1\), \(P = 0.008\), odds ratio = 0.58 [95% CI: 0.38–0.89]; corrected \(\chi^2 = 6.9\), \(P = 0.008\)). Corrected \(\chi^2\) and \(P\) values indicate stratified logistic regression analyses (LOGXACT; Cytel Software, Cambridge, MA) to adjust for ethnic heterogeneity.
cycles, using 100 ng of DNA, 3 m

individual genotypes were compared using CGGTTTTGTGTCTGGGAT-3

Results

Statistical Analysis

Group

Table 2. 5′ promoter microsatellite of NRAMP1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>165</td>
</tr>
<tr>
<td>Tuberculoid</td>
<td>105</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>122</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%). Allele 1 was rare and found in only 2 leprosy cases, as the 1/3 genotype, and allele 4 was absent. Genotypes were assigned according to size of microsatellite length variant in the NRAMP1 promoter [7]. There is no significant difference in genotype frequency between groups with or without stratification for ethnic group.

Exon 2 polymorphism. Forward primer of sequence 5′-GTG-ACAAAGGCTCCCCAA-3′ and HEX-labeled reverse primer 5′-CGGTTTTGTGTCTGGGAT-3′ were used to amplify a region of ~147 base pairs. The PCR cycle conditions were 94°C for 1 min, followed by 94°C for 10 s, 58°C for 20 s and 72°C for 30 s for 30 cycles, using 100 ng of DNA, 3 mM MgCl₂, 0.3 mM dNTPs, 0.1 μM each primer, and 1 U of Taq polymerase (AmpliTaq Gold) in a 15-μL reaction.

Statistical Analysis

This was performed in a stepwise manner, initially comparing overall genotype frequencies in each of the 3 groups using a 3 × 2 χ² test and then, if a significant overall difference between cases of leprosy of either type and controls was detected (P < 0.05), individual genotypes were compared using 2 × 2 χ² analysis.

Results

Allele frequencies for both VDR and NRAMP1 variants were initially compared between the various ethnic groups in the study and no statistically significant differences were observed (data not shown).

Analysis of the TaqI polymorphism in the 5′ region of the VDR gene showed that the overall distributions of genotypes between the groups studied (table 1) were different. In tuberculoid leprosy, the tt genotype was found at a significantly higher frequency (P < 0.001, odds ratio [OR] = 3.22, 95% confidence interval [CI] = 1.47–7.13) than in the controls. In contrast, the TT genotype was found at increased frequency in the lepromatous leprosy group compared with the controls (P = 0.3, OR = 1.67, 95% CI = 1.02–2.75). Heterozygotes of genotype Tt were found less frequently in both leprosy types than in controls (for leprosy vs. controls: P < 0.01, OR = 0.58, 95% CI = 0.38–0.89). Stratified logistic regression analyses (table 1) were carried out to adjust for ethnic heterogeneity using LOGXACT for Windows (Cytel Software, Cambridge, MA).

For the NRAMP1 promoter polymorphism, as elsewhere [14], alleles 2 and 3 were predominant. Comparison of the genotype (table 2) and allele frequencies between both leprosy types and controls showed no significant differences. The exon 2 polymorphism was absent in this population, as all the patients and controls typed were monomorphic, with only one allele of 147 bp. The other two polymorphisms, that in intron 4 (table 3) and another resulting from the TGTG deletion (table 4) in the NRAMP1 gene did not indicate over- or underrepresentation of any of the alleles in the groups studied. Heterozygotes for the TGTG deletion were absent in this population. All genotypes were in Hardy-Weinberg equilibrium.

Discussion

The association between VDR genotype and lepromatous leprosy observed in this study provides further evidence for the proposal that the vitamin D receptor may be an immune response gene regulating susceptibility to infectious disease in humans. There is epidemiologic evidence linking vitamin D deficiency and susceptibility to tuberculosis and in vitro data indicating an effect of the active metabolite of vitamin D, 1,25D₃, on mycobacterial growth. Addition of physiologic concentrations of 1,25D₃ impairs growth of Mycobacterium tuberculosis in human macrophages and monocytic cell lines [20]. The mechanism remains unclear but may relate to the increased differentiation and cytotoxicity observed in macrophages treated with 1,25D₃. Such in vitro studies are not possible for Mycobacterium leprae, but early studies of the treatment of leprosy with medications containing vitamin D are consistent with a possible immunomodulatory effect on this bacterium [21]. This case-control study identifying associations of VDR with leprosy and leprosy type in Bengalis provides evidence for a modulatory effect of the VDR pathway on leprosy as well as further data supporting the functional relevance of variation in or near the VDR gene. However, evidence of interpopulation heterogeneity in the association of osteoporosis with VDR genotype [22] suggests that variability may also be observed in associations with infectious diseases, perhaps related to variation in calcium intake or other gene-environment interactions, and studies in other populations will be required to determine the generality of these VDR-leprosy associations.

The differential association of VDR genotype with leprosy type is of particular interest in relation to the genetic control of cellular and humoral immune responses. Leprosy provides a model for understanding human immune responses to infection in that the disease presents as a spectrum in which the clinical manifestations correlate with the level of cell-mediated immunity to the bacterial pathogen. Tuberculoid leprosy pa-

Table 3. Intron 4 G to C polymorphism in NRAMP1.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>162</td>
</tr>
<tr>
<td>Tuberculoid</td>
<td>100</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>120</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%). No significant difference was observed between either leprosy group and control. Overall, χ² = 1.47, P = 0.8. Paired comparisons of genotypes between clinical groups showed no significant differences with or without stratification for ethnic group.
tients have a strong cellular immune response with skin lesions that contain well-organized tuberculoid granulomata with very few bacilli. In contrast, lepromatous leprosy patients have a stronger humoral immune response but a weak cellular response with bacilli-laden macrophages. Increasing evidence indicates that tuberculoid leprosy is associated with a predominantly TH1 type pattern of cytokine production in T cells from skin and peripheral blood, and, conversely, lepromatous leprosy is associated with a more Th2-shifted pattern of cytokine production [23, 24]. There is considerable interest in identifying this Th1-Th2 shift in humans may be VDR genotype, with tt homozygotes tending to produce a TH1-type immune response and TT homozygotes producing a Th2-type response. This possibility is compatible with the observation that tt homozygotes have been found to be resistant to pulmonary tuberculosis and to clear HBV infection more frequently (unpublished data); for both of these infectious diseases, a TH1-type immune response is probably of most protective importance. Of interest, in leprosy, the tt genotype is associated with tuberculoid type characterized by a strong cellular response but not with resistance to leprosy per se, which was associated with heterozygosity for VDR genotype.

In the case of NRAMP1, no significant association was obtained between the leprosy types and any of the four polymorphisms typed. However, the NRAMP1 variants studied were relatively poorly informative in this population. The lack of association with the NRAMP1 polymorphisms in this population does not rule out an association with the gene in other populations ethnically different from this or a weak association that would not be detected in a study of this size. Recent studies on tuberculosis and NRAMP1 polymorphism have shown some of the variants studied here to be associated with susceptibility to tuberculosis in The Gambia [18]. The results of this study, however, are consistent with two small family studies of leprosy from Polynesia and Pakistan, which found no evidence of linkage to the NRAMP1 region [16, 25].

These data suggesting that VDR genotype may influence the Th1-Th2 pattern of immune response in leprosy encourage assessment of the role of this genetic locus in a wide variety of human infectious and autoimmune diseases.

Acknowledgments

We thank V. N. Bhatia, Serology Institute, Calcutta, for DNA extraction facilities, and R. Bellamy and D. Kwiatkowski for discussions and advice.

References


Table 4. TGTG deletion in NRAMP1.

<table>
<thead>
<tr>
<th>Group</th>
<th>TGTG/TGTG</th>
<th>TGTG/deleted</th>
<th>Deleted/deleted</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>138 (89.6)</td>
<td>16 (10.4)</td>
<td>0</td>
<td>154</td>
</tr>
<tr>
<td>Tuberculod</td>
<td>95 (91.3)</td>
<td>9 (8.7)</td>
<td>0</td>
<td>104</td>
</tr>
<tr>
<td>Lepromatous</td>
<td>105 (89.0)</td>
<td>13 (11.0)</td>
<td>0</td>
<td>118</td>
</tr>
</tbody>
</table>

NOTE: Data are no. (%). No significant difference was observed between either leprosy group and controls. Overall, χ² = 0.36, P = .8. Paired comparisons of genotypes between clinical groups showed no significant differences with or without stratification for ethnic group.


