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Susceptibility to Leprosy May Be Conditioned by an Interaction between the NRAMP1 Promoter Polymorphisms and the Lepromin Response[1, 3]

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ABSTRACT

Controversial results have been achieved by attempting to associate the NRAMP1 gene with *Mycobacterium leprae* susceptibility as well as with the Mitsuda reaction, which represents a specific immune response to *M. leprae*. This study evaluated this association as well as the interaction of the polymorphism $(GT)_n$ in the promoter region of the NRAMP1 gene with a specific immune response to *M. leprae* measured by the intradermal Mitsuda test in leprosy patients and in non-consanguineous household contacts. The study aimed to evaluate the association of this gene polymorphism with resistance or susceptibility to the disease, and/or with clinical forms of the disease, in a population in an endemic area served by the State Reference Center in Sanitary Dermatology and Leprosy , Federal University of Uberlandia, MG, Brazil. Leprosy patients (90) were diagnosed according to Ridley and Jopling criteria and they grouped into multibacillary (MB) and paucibacillary (PB) patients. The control group consisted of 61 non-consanguineous contacts. NRAMP1 promoter genotypes were obtained through amplification by the polymerase chain reaction (PCR) followed by the detection through the low ionic-strength single strand conformational polymorphism (LIS-SSCP) electrophoretic technique. There were no significant differences in the allelic and genotypic frequencies for alleles 2, 3, and 4 in relation to the Mitsuda test among patients and household contacts, nor between those with MB and PB forms. However, individuals with a negative lepromin response associated with genotypes 22 and 23 presented a 7- and 8-fold greater chance of developing leprosy, respectively. Therefore, the NRAMP1 gene promoter polymorphism exhibited an interaction with the lepromin response, suggesting that allele 2 of the NRAMP1 promoter is an independent genetic factor that predisposes cells to enable pathogen survival, probably due to its low efficiency in iron transport. However, establishment of the infection and disease development may be conditioned by other immunological and genetic factors.

RÉSUMÉ

Des résultats controversés ont été obtenus lorsque la relation entre le gène NRAMP1 et la susceptibilité à *Mycobacterium leprae* ou bien la réaction de Mitsuda, qui représente une réponse immunitaire spécifique contre *M. leprae*, a été étudiée. Cette étude a évalué l'association et également l'interaction entre le polymorphisme $(GT)_n$ de la région promotrice du gène NRAMP1 et la réaction immunitaire spécifique contre *M. leprae*, mesurée par le test intradermique de Mitsuda chez des patients lépreux et chez des personnes non-consanguines en contact de ces patients. Le but de cette étude était d'évaluer l'association de ce polymor-

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phisme génétique avec la résistance ou la susceptibilité à la maladie, et/ou avec sa forme clinique chez une population où la lèpre est endémique, qui est suivie par le Centre de Référence d'Etat en Dermatologie Sanitaire et Lèpre, Université Fédérale de Uberlandia, MG, Brésil. Les patients hanséniens (90) furent diagnostiqués selon les critères de Ridley et Jopling et regroupés en patients multibacillaires (MB)et paucibacillaires (PB). Le groupe contrôle comprenait 61 personnes contactes non-consanguines. Les génotypes de la région promotrice du gène MRAMP1 furent obtenus en détectant le polymorphisme de conformation de simple brin en milieu de faible force ionique (LIS-SSCP) après amplification par la réaction de polymérisation en chaîne (PCR). Aucune différence significative ne fut obtenue entre les fréquences d'allélismes et de génotypes pour les allèles 2, 3 et 4 et les tests de Mitsuda parmi les patients et les contacts, ainsi qu'entre les formes MB et PB. Les individus présentant une réaction négative à la lépromine, associés au génotype 22 et 23, présentaient un risque 7 et 8 fois plus grand de développer la lèpre, respectivement. Donc, une interaction entre le polymorphisme de la partie promotrice du gène NRAMP1 et la réponse à la lépromine existe, ce qui implique que l'allèle n°2 du promoteur de NRAMP1 est un facteur génétique indépendant qui prédispose les cellules à la survie du pathogène, probablement causé par la faible efficacité de ces cellules dans le transport du fer. Cependant, l'infection primaire par le bacille et le développement de la maladie sont probablement liés à d'autres facteurs immunologiques et génétiques.

RESUMEN

Cuando se ha tratado de asociar el gene de susceptibilidad a *Mycobacterium leprae* NRAMP1, con la reacción de Mitsuda, se han encontrado resultados controversiales. La reacción de Mitsuda representa una respuesta inmune específica hacia *M. leprae*. En este estudio se evaluó tal asociación y la interacción del polimorfismo *(GT)n* en la región promotora del gene NRAMP1 con el resultado de la reacción de Mitsuda en pacientes con lepra y en contactos domésticos no consanguíneos. El objetivo del estudio fue evaluar la asociación de este polimorfismo génico con la resistencia o susceptibilidad a la enfermedad, y/o con la forma clínica de la misma en una población de una área endémica de Brasil atendida por el Centro Estatal de Referencia en Dermatología Sanitaria y Lepra de la Universidad Federal de Uberlandia, MG. Los pacientes con lepra (90), diagnosticados de acuerdo a los criterios de Ridley y Jopling, se agruparon como multibacilares (MB) y paucibacilares (PB). El grupo control consistió de 61 contactos domésticos no consanguíneos. Los genotipos del promotor de NRAMP1 se obtuvieron por la reacción en cadena de la polimerasa (PCR) y por la técnica electroforética LIS-SSCP (*low ionic-strength single strand conformational polymorphism*).

No se observaron diferencias significativas en las frecuencias alélicas y genotípicas para los alelos 2, 3, y 4, en relación a la prueba de Mitsuda entre los pacientes y contactos domésticos, ni entre los pacientes con las formas MB y PB. Los individuos con respuesta negativa a la lepromina asociados a los genotipos 22 y 23 presentaron, respectivamente, 7- y 8-veces más probabilidad de desarrollar la enfermedad. Por lo tanto, observamos que el polimorfismo en el gene promotor de NRAMP1 presentó una interacción con la respuesta a la lepromina, sugiriendo que el alelo 2 del promotor de NRAMP1 es un factor genético independiente que predispone, en las células, la supervivencia del patógeno, probablemente debido a su baja eficiencia en el transporte de hierro; sin embargo, el establecimiento del bacilo y el desarrollo de la enfermedad pueden ser condicionadas por otros factores inmunológicos y genéticos.

Leprosy, caused by *Mycobacterium leprae,* is a chronic disease that afflicts over 620,000 new cases per year, and most of these patients are found in India and in Brazil ([38]). The expression of the disease results from the interaction between the bacillus and the immunological system in such a way that most people infected develop an effective immune response against the bacillus without the presence of the disease, while others exhibit a spectrum of clinical manifestations intimately related to the immunological patterns of the host response to the pathogen ([11, 12]).

At one end of the clinical spectrum is tuberculoid leprosy (TT), in which bacterial growth is limited by a vigorous cellular immune response with a predominance of the CD4+ cells and the Th1-type cytokines (IL-2 and INF-γ) in the lesions of the skin. At the

opposite end is lepromatous leprosy (LL), in which the cytokine pattern found in the lesions is of the Th2-type (IL-4, IL-5 and IL-10), and $CD8^+$ cells occur predominantly, along with a strong but inefficient humoral response ([19, 40]).

The Mitsuda test, which consists of the intradermal injection of a suspension of heat-killed *M. leprae*, has been used as a measure of the cell-mediated immune response to the bacillus. The injection of the bacillus produces a positive local intradermal reaction in patients with tuberculoid leprosy, while lepromatous patients do not develop an intradermal reaction in response to the bacillus. From a clinical standpoint, the test is an important indicator of cellular immunity to *M. leprae*. In patients with leprosy it is considered to be of good prognostic value for resistance when positive, and for susceptibility when negative, and in normal individuals a positive test is associated with a smaller risk of developing the disease ([15]). Although individuals with a negative Mistuda response present a higher risk of developing lepromatous leprosy, some may never develop the disease, demonstrating that the relationship between Mitsuda reactivity and resistance is not fully established ([15]).

The origin of the inefficient immune response in individuals with multibacillary leprosy is uncertain, but the high rate of correspondence of leprosy type between identical twins favors the hypothesis of an association with genetic factors ([16]). The polymorphisms of the major histocompatibility complex (MHC) ([20]) and other genes not related to the MHC, such as the gene of the macrophage protein associated with natural resistance (NRAMP1, now known as SLC11A1) ([9, 21]), vitamin D receptor gene (VDR) ([31]), TAP1 and TAP2 genes ([25]), have been associated with susceptibility to leprosy. Other loci, such as the tumoral necrosis factor (TNF) gene, linked to the HLA region, have also been reported to be able to determine the subtypes of leprosy ([32, 34]).

The NRAMP1 gene is composed of 15 exons and is located on human chromosome region 2q35, and covers at least 16 kb of DNA. It encodes a protein of 550 amino acids, the human Nramp1 integral membrane protein, that is found exclusively in the lysosomal compartment of monocytes and macrophages ([9]).

Over 10 polymorphic sites have been described in the NRAMP1 gene. In the promoter region of this gene, whose polymorphisms are functional since they affect the expression of the Nramp1 protein, 4 alleles were identified (1, 2, 3, and 4) ([5]). Alleles 2 and 4 are described as poor promoters of NRAMP1 and consequently, could offer protection against auto-immune diseases but would increase the susceptibility to infections by intracellular parasites. Allele 3 could yield larger expression of the Nramp1 protein, favoring protection against intracellular parasites, but it could also be involved in auto-immune syndromes ([6]).

Various studies have been carried out in an attempt to establish a connection between the polymorphisms of the NRAMP1 gene and susceptibility/resistance to autoimmune diseases ([4, 7, 29, 36]) and to diseases caused by parasites, such as *Salmonella typhimurium, Leishmania donovani,* and *Mycobacterium bovis* ([37]).

One of the strategies to identify genomic regions associated with disease-causing genes is through genetic linkage studies based on a statistical estimate of whether two loci are likely to lie near each other, called the LOD score (decimal logarithm of an odds ratio: the odds that two loci are linked with recombination fraction divided by the odds that the two loci are unlinked, recombination fraction 0.5) ([10, 22]). In the traditional LOD scores, the boundary value of 3 it has been used to indicate linkage, and –2 for exclusion. Another strategy is the use of odds ratio (OR) and its confidence interval (CI) to indicate a chance for an event occurrence based on the variation of two categorical variables, with values of 0 and 1 (negative and positive results). The odds ratio can be interpreted as a measure of the magnitude of association between two raters. Calculating the standard error of log (OR), one can easily test the significance of log (OR) and/or construct confidence intervals ([2]).

Using LOD scores, haplotypes of the NRAMP-1 gene promoter region were associated with susceptibility to tuberculosis ([5]). Linkage studies between resistance to leprosy and polymorphisms of the NRAMP1 gene have not found any association of gene

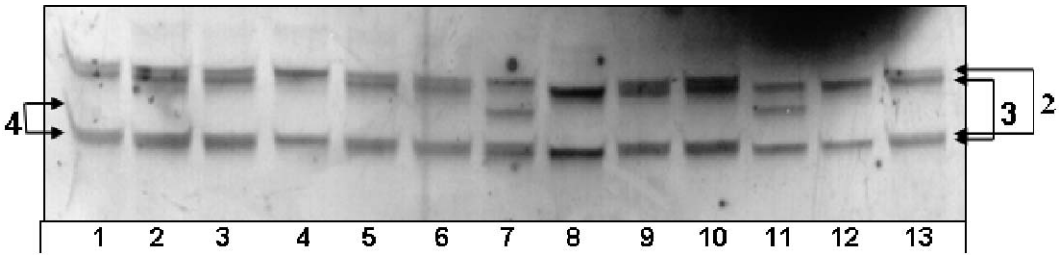

FIG. 1. PAGE LIS-SSCP gel stained by silver. Lane 4 is from a patient of genotype "22"; lanes 1, 2, 3, 5, 6, 9, 10 and 13 are from patients with "23" genotype, lanes 8 and 12 are from patients with the "33" genotype, lane 7 is from "24" genotype, and lane 11 is from a "34" individual.

haplotypes with susceptibility to the disease in the French Polynesia ([30]). However, a co-segregation pattern was observed between the two proximal markers to the NRAMP1 gene, a microsatellite and a single nucleotide polymorphism (SNP), and susceptibility to leprosy ([1]). On the other hand, a linkage between the NRAMP1 gene and a positive reaction to the Mitsuda test was demonstrated in pairs of consanguineous individuals, regardless the presence or absence of leprosy ([3]). Another similar study has found no association between this gene and the Mitsuda test ([16]).

Consequently, the results obtained in the latest studies proved to be insuf ficient for an implication of the NRAMP1 gene with the susceptibility and/or resistance to leprosy, making it important to conduct further studies that may confirm the influence of this gene. This study evaluated the association as well as the interaction of the polymorphism $(GT)_n$ in the promoter region of the NRAMP1 gene with a specific immune response to the *M. leprae* measured by the intradermal Mitsuda test, in leprosy patients and in non-consanguineous household contacts, aiming to evaluate the association of this gene with resistance or susceptibility to leprosy, and/or with clinical form of leprosy.

SUBJECTS AND METHODS

Subjects. Leprosy patients (90) and their household contacts (61) from the State Reference Center of Sanitary Dermatology and Leprosy of the Federal University of Uberlândia, (UFU), Minas Gerais (MG), Brazil, were invited to take part in this study, under the approval of the UFU Research Ethical Committee. Leprosy patients were diagnosed and classified according to the criteria of Ridley and Jopling ([28]), considering: clinical examination, histopathology of skin lesions, Mitsuda test, and the bacilloscopic index (BI) ([27]). Patients with undetermined types of leprosy were not considered.

For genetic analysis, we have used an adaptation of the WHO classification of patients ([39]), without considering the number of lesions, but taking into account the lesions characteristics and the bacilloscopic index. Therefore, patients were grouped into paucibacillary forms (45 patients), which consisted of tuberculoid (TT) and borderline-tuberculoid (BT) patients with negative bacilloscopy, and into multibacillary forms (45 patients), with mid-borderline (BB), borderline-lepromatous (BL) and lepromatous (LL) patients, who had a positive bacilloscopy.

The group considered as control was ensured by careful dermatological examination, and was composed of non-consanguineous household contacts who had similar exposure to *M. leprae*, but without symptoms or signs of the disease.

Mitsuda test. The Mitsuda antigen (suspension containing 6.0×10^7 bacilli/ml, heat-killed), supplied by the Instituto Lauro de Souza Lima (ILSL, Bauru—SP), was injected intradermally (0.1 ml) at the upper third of the anterior face of the right forearm. The readings were performed by an experienced leprosy specialist 28 days after inoculation. The results were measured in millimeters and grouped for quantitative and qualitative analysis ([15]). However, for a genetic analysis using categorical data, all

TABLE 1. *Allelic frequencies of the NRAMP1 gene promoter polymorphisms in leprosy patients, classified in paucibacillary and multibacillary forms, and household contacts of the State Reference Center of Sanitary Dermatology and Leprosy, UFU/SUS, Uberlândia, Minas Gerais, Brazil.*

Groups	Allelic frequency			
	2	3	4	N
Household contacts	0.369	0.574	0.057	61
Paucibacillary patients	0.333	0.600	0.067	45
Multibacillary patients	0.322	0.656	0.022	45

N = number of subjects.

patients and contacts were classified into two classes, according to their response pattern: "negative" for readings <7 mm, which consisted of negative and weak positive (+) reactions; and "positive" for readings ≥7 mm, which consisted of positive (++) and strong positive (+++) reactions or with the presence of fluctuant swelling and/or ulcerations.

Genotyping for the polymorphism on the NRAMP1 promoter gene. The DNA was extracted using the phenol:chloroform method described previously ([33]), with some modifications, and its quality was analyzed in agarose gels stained with ethidium bromide.

The amplifications through the PCR used the direct primer , 5′-CTCGCATTAGGCCAACGA and reverse 5′-TTCTGTGCCTCCCAAGTTAGC ([31]). The conditions for PCR were 35 cycles at 95°C for 40 sec, 58°C for 40 sec and 72 °C for 50 sec, preceded by initial denaturation of 95 °C for 5 min and final extension at 72°C for 10 min. The reactions occurred were performed in a volume of 25 µl containing from 50 to 200 ng of genomic DNA, 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 7 pmol of each primer and 1.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbas-CA).

The alleles 2, 3, and 4, containing 200, 198, and 188 base-pairs, respectively, were visualized in PAGE LIS-SSCP gel, 14% acrylamide:bis (49:1), at room temperature, with 10 V/cm for 24 hrs, followed by silver nitrate staining (Fig. 1). All polymorphic bands, representing all three alleles, were excised from the gel and cloned (TOPO-TA Cloning kit, Invitrogen, Carlsbas-CA) for confirmation of allelic sizes through dideoxy cycle sequencing. Allele 4 was also confirmed by the molecular weight previously described ([13]). The LIS-SSCP strategy allowed a fast and reliable characterization of the (GC)n promoter variation of the NRAMP1 gene, without using radioactivity and/or restriction endonucleases.

Statistical analysis. The statistical analyses were developed in SAS version 6.11 (1993) and Prophet version 5.0 (1996) programs. Comparisons of allelic frequencies of the NRAMP1 promoter polymorphisms among groups were evaluated by the *t* test. The Hardy-Weinberg Equilibrium for the NRAMP1 promoter polymorphisms in the population was tested by the χ^2 test. Regression analysis was performed to determine the association among all possible alleles and the Mitsuda test. Results of Mitsuda test means among groups were evaluated across NRAMP1 genotypes through analysis of variance and *t* tests. The association of the NRAMP1 genotypes and the Mitsuda test was verified by Pearson's correlation and the χ^2 test. Odds ratios were determined for each genotype, Mitsuda tests (negative versus positive) and their interaction, comparing controls versus leprosy patients, to calculate the chance of developing leprosy.

RESULTS

The allelic frequencies of the polymorphisms for the NRAMP1 gene promoter analyzed by the chi-square test were not significantly different among groups (household contacts, paucibacillary and multibacillary patients) (Table 1). Frequencies of alleles 2 and 3 have been described elsewhere ([6]) to be 0.25 and 0.75, respectively. However, in this study , allele 2 presented higher frequencies, which varied from 0.32 to 0.36.

TABLE 2. *Mean and standard deviation of the Mitsuda test result for leprosy patients and household contacts, according to the NRAMP1 promoter genotype, State Reference Center of Sanitary Dermatology and Leprosy, UFU/SUS, Uberlândia, Minas Gerais, Brazil.*

NRAMP1 genotype	Mean and standard deviation of the Mitsuda test											
	HC			PB patients			MB patients			Total		
	N	M	S.D.	N	M	S.D.	N	M	S.D.	N	M	S.D.
22	8	6.6	2.2	4	6.3	6.3	4	0.8	1.5	16	5.1	4.1
23	24	8.6	3.4	18	5.6	2.9	20	0.4	1.0	62	5.2	4.4
33	22	6.5	3.6	17	6.1	3.8	20	0.0	0.0	59	4.2	4.3
24/34/44	7	7.0	4.2	6	8.7	2.7	2	0.0	0.0	15	6.7	4.3
Total	61	7.4	3.5	45	6.3	3.7	46	0.2	0.8	152	4.9	4.3

N = number of patients or contacts; M = mean; S.D. = standard deviation in millimeters; HC = healthy contacts PB = paucibacillary; MB = multibacillary. Differences in *t* tests were the following: HC vs. PB ($t = 1.56$; $p = 0.06$); HC vs. MB ($t = 4.86$; $p < 0.00001$), and PB vs. MB ($t = 10.8$; $p < 0.00001$).

Allelic and genotypic frequencies presented no statistical significance compared to those described in a previous study ([37]). However, significant dif ferences were detected between alleles 2 and 3 within each group evaluated ($p = 0.0017$), with significantly lower frequencies of allele 2, 0.369, 0.333, and 0.322, for household contacts, PB and MB patients, respectively, in comparison to allele 3, which presented frequencies of 0.574, 0.60, and 0.656, respectively.

The mean value of Mitsuda test of household contacts (7.4) was greater than that observed in paucibacillary and multibacillary patients (6.3, and 0.2, respectively) supporting the association of disease resistance with the positive Mitsuda test. However , there were no dif ferences among Mitsuda test means across NRAMP1 genotypes (Table 2). The Mitsuda test values were further transformed into qualitative results, classifying groups as negative and positive, as described. No association was found between NRAMP1 promoter genotypes or alleles and the Mitsuda test through regression analysis, using either quantitative or qualitative data. However , an interaction between them favoring leprosy occurrence was demonstrated through odds ratio analysis (Table 3).

Analysis of Mitsuda test results among household contacts (HC), PB and MB patient groups revealed significant differences ($p < 0.0001$), except between contacts and PB, which rvealed no differences ($p = 0.06$) (Table 2).

The bacterial index (BI) results for patients were compared among all NRAMP1 gene promoter alleles and no significant differences were detected for this variable among NRAMP1 promoter genotypes.

Odds ratios were calculated for each genotype in all combinations considering the leprosy patients and control groups, and very low values (under 1.0) were obtained, with non-significant statistical confidence intervals. The odds ratio for negative Mitsuda and disease status (leprosy occurrence) was 4.65 ($\chi^2 = 17.26$; $p < 0.0001$).

The lack of association between NRAMP1 genotypes and the Mitsuda test may lead us to consider them as two independent events. Therefore, the interaction of the NRAMP1 promoter genotypes and the Mitsuda response was further investigated, considering these as independent factors that could interact favoring disease establishment. The odds ratios were obtained for each genotype, comparing the Mitsuda response between leprosy patients and household contacts (Table 3). Results were highly significant for genotype 23 (OR = 8.09; CI 95%: 2.55 to 25.64) and for the combination of genotypes 22 and 23 (OR = 7.06; CI 95%: 2.57 to 19.39) when individuals presented a negative Mitsuda test. Genotype 22 presented a very high odds ratio (7.0) with a marginal confidence interval close to significance.

DISCUSSION

The present study has investigated the polymorphism in the promoter region of the NRAMP1 gene due to the strong evidence that such variation may be associated with the regulation of the gene, which is directly

TABLE 3. *Chance of developing leprosy disease (odds ratios) by associating the NRAMP1 promoter genotypes [5′(CT)n] and Mitsuda test results in leprosy patients and household contacts (control), State Reference Center of Sanitary Dermatology and Leprosy, UFU/SUS, Uberlândia, Minas Gerais, Brazil.*

Genotype(s)	Groups	Mitsuda test (number of individuals)†		Odds Ratios (CI = 95%)‡
		Positive	Negative	
22	Control	4	4	7.00 (0.56–86.82)
	Leprosy	1	7	
23	Control	17	7	8.36 (2.64–26.45)*
	Leprosy	9	30	
33	Control	9	13	2.51 (0.79–7.97)
	Leprosy	8	27	
22 + 23	Control	21	11	7.25 (2.64–19.89)*
	Leprosy	10	37	

† Mitsuda test results: positive (37 mm) and negative (<7 mm).
‡ Odds ratio followed by * indicates statistical significance. CI = confidence interval.

associated with susceptibility to infectious disease ([6]). The results have demonstrated that the prevalence of alleles 2, 3, and 4 of the NRAMP1 gene promoter among groups (household contacts, paucibacillary and multibacillary leprosy patients) of this endemic area was not significantly different from other populations, although the frequency of the unfavorable allele 2 was 7% to 11% higher than that observed elsewhere ([6]). The higher prevalence of allele 2 in this population may be an indication that the local environment could be favoring or selecting for a higher frequency of this allele. However, it is believed that there may be a balanced selection between alleles 2 and 3 due to their probable association to infectious and autoimmune disease susceptibility, respectively ([6]).

This study focused on the polymorphism in the promoter region of the NRAMP1 gene in order to investigate the hypothesis that this polymorphism would be associated with the degree of the expression of the NRAMP1 gene, as proposed by previous studies ([6, 8]), and that the higher expression of the NRAMP1 gene would result in a more vigorous response to the Mitsuda antigen, with corresponding protection to the multibacillary forms of leprosy. However, no correlation was obtained between NRAMP1 genotypes and the Mitsuda response in this study, corroborating with results of other studies ([16, 31]). Similarly, association was observed with the different forms of leprosy, as demonstrated previously ([27]). On the other hand, the 4-bp polymorphism in the NRAMP 3′-untranslated region, detected in another study ([18]), was not associated to leprosy *per se,* but it was associated with one form of leprosy. Therefore, the contradictory results regarding this polymorphism were not able to confirm the possible influence of the NRAMP1 polymorphisms on the clinical presentation of leprosy.

The first evidence indicating an association of the NRAMP1 gene with leprosy was obtained in a segregation study of consanguinous pairs in 20 ethnic Chinese and Vietnamese family groups, where a significant non-random segregation was found for the haplotypes of the NRAMP1 gene among the consanguineous pairs ([1]). Additionally, in the segregation analysis according to the LOD score, the NRAMP1 gene haplotypes were associated with susceptibility to tuberculosis ([10]) and with positive results of the Mitsuda test ([3]).

The latter study ([3]), associated to a previous study of the same families ([1]), has demonstrated a linkage of the chromosome 2q35 locus with Mitsuda response, which has only identified a candidate region where a gene is located near or at the locus controlling the Mitsuda response. However, in that investigation ([3]), a possible confounding effect of disease status was shown on the linkage test, which is highly influenced by the Mitsuda response. It is also important to notice that the small data set of consanguinous sib-pairs may have suffered an im-

portant genetic bias due to the allele fixation in a very small number of families, which favors allele coincidence, where sibs have a 75% probability of sharing at least one allele. This is supported by the ethnic background: significant dif ferences were observed only for the Vietnamese population ([3]), but not for Chinese ([3]), Brazilian ([16]), and Indian ([31]) populations. Since the power of LOD tests for establishing linkage or exclusion is decreased with small data sets, as demonstrated elsewhere ([24]), it is possible that the linkage shown with the quantitative Mitsuda trait ([3]) may be only a trend which indicates a possible interaction with other candidate immunological and genetic factors. Another important confounding effect is the period and frequency of contact with infected patients and the time required for disease development, which can not be recorded or accurately estimated, producing a false positive linkage due to genotypic disequilibrium in small populations. Contradictory Mitsuda test results may further complicate this analysis due to the presence of positive Mitsuda individuals that develop disease, as well as negative Mitsuda individuals that will never develop leprosy , suggesting that other factors may be contributing to the disease development. In fact, there are very few linkage studies firmly established for quantitative traits in humans, especially because statistical methods for exclusion are still underdeveloped ([24]).

In our study, we have genotyped 90 patients and 61 non-consaguinous household contacts. This population sample is a true representation of allelic and genotypic frequencies of the Brazilian population that is in Hardy-W einberg equilibrium. Dif ferences between our study and results obtained elsewhere ([3]) are related to the consanguinity status and the number of patients representing families. Our study represents 90 different families, while the earlier studies represent 20 nuclear families. However, the allelic and genotypic frequencies distribution in these two populations could not be compared since no genotypic information was provided on the study with 4 Chinese and 16 Vietnamese families ([3]), nor was possible to verify the frequency of the other microsatellite loci and their linkage with the Mitsuda tests.

An earlier study of 30 individuals (22 healthy and 8 leprosy patients) did not detect any association of the loci 274C/T , D543N and 1729 of the NRAMP1 gene with the positive reaction to the Mitsuda test ([16]). Additionally, the LOD score tested in 7 family groups in French Polynesia, including 39 leprosy patients and 45 healthy individuals, was not significant for NRAMP1 haplotypes and leprosy association ([30]). Another investigation also failed to detect an association of the promoter $(GT)_n$ region, the 274C/T and the TGTG deletion with the lepromatous and tuberculoid forms of leprosy ([31]). All these observations are in agreement with this investigation, which has not found any association between NRAMP1 promoter polymorphisms with leprosy *per se,* nor with Mitsuda tests or with leprosy types. However, the NRAMP1 gene may have influence over the pathogenicity of leprosy, since there is increasing evidence favoring the linkage of the locus NRAMP1 with tuberculosis, which is also a disease in which macrophage infection is important ([14]).

Although there is a high association of the positive Mitsuda test with leprosy resistance, the high variability of these tests in household contacts and PB patient's groups observed in this study supports the hypothesis that resistance to leprosy is conditioned by multiple genes ([23, 26]), and these genes may be masking the true ef fect of the NRAMP1 gene in the disease outcome.

No interaction between Mitsuda tests and NRAMP genotypes has previously been investigated relating the two variables to leprosy. This is the first study that uses combined data of NRAMP1 genotypes and Mitsuda tests classes to estimate the chance of developing leprosy . The odds ratio for negative Mitsuda and leprosy occurrence was highly significant (OR = 4.65) as shown elsewhere ([15]) corroborating the close association of negative Mitsuda results and leprosy susceptibility. On the other hand, the odds ratio for allele 2 and leprosy occurrence was non-significant (OR = 0.88; $p > 0.05$).

However, in this study we have also shown a very important interaction between NRAMP1 gene and Mitsuda tests. The lack of association between the two variables may lead us to consider them as two independent events that may interact with each

other. The NRAMP1 promoter genotypes 22 and 23 were found to be unfavorable genotypes when present in combination with a negative Mitsuda response, showing an approximately 7-fold greater chance of developing leprosy disease. The high odds ratio for genotype 22 (7.0) with a confidence interval not quite reaching significance (probably due to the low number of individuals evaluated in this class, provides an indication that this genotype may also present the same significant tendency of genotype 23) suggests that a dominance of allele 2 favors pathogen survival. Consequently, the susceptibility phenotype could be determined by the association between autologous factors such as low-activity of the NRAMP1 gene promoter alleles, point mutations and/or deletions with influence upon the transporting function of iron ions of the NRAMP1 protein, and several exogenous factors. These results are supported by other studies ([20, 21]) which suggest that susceptibility to leprosy is multigenic, with a high heterogeneity among dif ferent populations studied. In this sense, a complex genetic model of susceptibility to leprosy could make detection of linkage difficult ([1]).

Recently, a complex segregation analysis and a genome-wide scan have demonstrated that the susceptibility to the disease itself or its progression may be related to dif ferent genetic factors. Siddiqui, *et al*. ([35]) identified a major susceptibility locus to leprosy (10p13) regardless of the polygenic nature of the disease. On the other hand, it was demonstrated that the segregation of the HLA/TNF region, locus 6p21, has a strong link to the development of the clinical forms of leprosy ([21]).

Mira, *et al*. ([20]) proposed a genetic model for leprosy susceptibility in two phases: susceptibility of the disease itself would be linked with non HLA genes such as the NRAMP1, in other words, genes that would control the progress of the infection until the development of the clinical symptoms of the disease, while the loci linked to the HLA would determine the subtypes of leprosy.

The hypothesis that the dif ferential NRAMP1 gene expression is related to *M. leprae* survival ([6]) instead of microbial proliferation ([17]) is supported by this study that demonstrated that there was no association of the NRAMP1 promoter genotypes and the bacilloscopic index. Hence, the low expression of the iron transport protein (NRAMP1), conditioned by the allele 2, may function in microbial persistence, especially in Mitsuda-negative individuals.

We propose that the NRAMP1 gene favors microbial survival, probably by transporting the iron inefficiently, but it will only affect the development of clinical symptoms in association with other immunological and genetic factors, as demonstrated by the interaction with a negative Mitsuda test. Therefore, leprosy susceptibility can not be conditioned by a unique gene; instead, it is a function of multifactorial host conditions that require at least two independent molecular events that interact to each other.

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