Granulomatous Reaction to Intradermal Injection of Lepromin (Mitsuda Reaction) Is Linked to the Human NRAMP1 Gene in Vietnamese Leprosy Sibships


The Mitsuda test, which measures the specific immune response against intradermally injected lepromin, has a high prognostic value for susceptibility or resistance to the lepromatous form of leprosy. A sib-pair linkage analysis between the Mitsuda response and the NRAMP1 gene was done among 20 nuclear families with leprosy (totaling 118 sibs) from Ho Chi Minh City, Vietnam. All family subjects were genotyped for several intragenic and flanking NRAMP1 markers, leading to the definition of a fully informative NRAMP1 haplotype. Significant linkage was observed between NRAMP1 and Mitsuda reaction when considered either as a quantitative \( (P < .002) \) or as a categorical \( (P = .001) \) trait. Separate analyses among healthy and affected sibs showed evidence for linkage in both subsamples, indicating that linkage between the Mitsuda reaction and NRAMP1 is independent of leprosy status. These results support the view that NRAMP1 plays a regulatory role for the development of acquired antimycobacterial immune responses as determined by in vivo Mitsuda test reaction.

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, with an annual incidence of \( \sim 500,000-600,000 \) cases worldwide [1]. The expression of the disease results from the interactions between the leprosy bacillus and the immune system of the infected host [2]. Whereas most infected persons develop an effective immunity without disease, others present a wide spectrum of clinical manifestations correlated with the immunologic response of the patient. At one pole of this spectrum, patients with tuberculoid leprosy show well-developed specific cellular responses and low levels of antibody to *M. leprae*, whereas the opposite is observed for patients at the other end of the spectrum, with lepromatous (multibacillary) leprosy. The so-called Mitsuda skin reaction, which measures the granulomatous immune response to intradermally injected heat-killed leprosy bacilli (lepromin), is an interesting indicator of efficient anti-leprosy immunity because it has a good prognostic value for susceptibility (when negative) or resistance (when positive) to the lepromatous form of the disease [3, 4].

The large variability of the host response to infection by the leprosy bacillus has been demonstrated to be genetically controlled in animal models. In particular, studies of inbred strains of mice have identified a gene (denoted as *Lsh/Ity/Bcg*) located on proximal mouse chromosome 1 that controls innate resistance to infection with several intracellular macrophage pathogens, including *M. lepraemurium* and *M. bovis* (bacille Calmette-Guérin [BCG]). This gene has been isolated by positional cloning [5] and was designated “natural resistance associated macrophage protein 1” (*Nramp1*). Subsequently, the human homologue *NRAMP1* was cloned [6], and 9 sequence variants within *NRAMP1* and several microsatellite markers in the chromosomal vicinity of the gene were described [7]. These markers were used to show that susceptibility to leprosy per se, that is, leprosy independent of its specific clinical manifestation, was closely linked to the *NRAMP1* genomic region in a sample of 20 Vietnamese multiplex leprosy families [8]. In a recent case-control study of a population from India, no association was found between 4 *NRAMP1* polymorphisms and lepromatous or tuberculoid leprosy [9]. However, the studied *NRAMP1* variants were poorly informative for this Indian population, and a genetic heterogeneity in susceptibility to leprosy cannot be ruled out, as already suggested [8]. In another study from the Gambia, a significant association of *NRAMP1* alleles with sus-
ceptibility to tuberculosis was observed [10]. These results suggest that \( NRAMP1 \) allelic variants could be risk modulators for the development of 2 major mycobacterial diseases in humans.

These previous studies have not determined at what level of the infectious process \( NRAMP1 \) is likely to exert its disease risk–modulator effect. In the mouse model, it has been well established that \( Nramp1 \) alleles profoundly influence the intrinsic capability of macrophages to resist infection by intracellular parasites [11, 12]. Hence, it is possible that infection and resulting disease of the host could be prevented exclusively on the level of the macrophage. On the other hand, it is also possible that \( NRAMP1 \) polymorphisms influence the quality and quantity of the antmycobacterial immune response and that susceptibility to the disease is the result of a less efficient \( NRAMP1 \)-modulated acquired immune response (reviewed in [13]). To address the question of whether \( NRAMP1 \) influences the acquired antmycobacterial immune response, we performed a linkage analysis between the \( NRAMP1 \) genome region and the extent of the Mitsuda skin reaction among individual members of families with leprosy.

**Subjects and Methods**

**Families.** The nuclear families studied correspond to the sample identified from records of the Dermatology Hospital in Ho Chi Minh City and used elsewhere to perform a linkage analysis of leprosy clinical status [8]. This family sample includes 20 nuclear families with at least 2 siblings with leprosy. The ratio of Vietnamese (16 families) to Chinese (4 families) reflects approximately the proportion of both ethnic groups in the registry.

**Phenotype definition.** All Mitsuda reactions were determined by experienced Vietnamese leprologists and were read 28–30 days after the intradermal injection of 0.1 mL of lepromin into the volar surface of the forearm [14]. Mitsuda reaction was measured for 118 offspring (including healthy offspring) belonging to 20 nuclear families with sibship sizes ranging from 2 to 12. For further analyses, the delayed lepromin reaction was first considered as a quantitative trait, ranging from 0 to 14 mm. We also coded the Mitsuda reaction as a 4-class categorical trait: < 3, [3–5], [5–10], and >10 mm. The cut points of 3 and 5 mm are defined following the recommendations of the Sixth International Leprosy Congress held in Madrid (1953), and the 10-mm cut point was later suggested by Languillon [15]. A Mitsuda reaction < 3 mm is classically considered as negative, whereas a reaction > 10 mm is clearly positive. We also collected information about leprosy status and clinical subtype, classified as either lepromatous (a category including the borderline lepromatous and polar lepromatous forms of the Ridley and Jopling [3] classification), tuberculoid (including polar tuberculoid and borderline tuberculoid forms), or indeterminate (all other subtypes).

**Haplotype definition and genotyping.** In the present study, we used the extended haplotype defined in [8] to follow the segregation of \( NRAMP1 \) in the sample families. This haplotype consists of 6 diallelic \( NRAMP1 \) polymorphisms, 1 restriction-fragment length polymorphism located within the \( TNPI \) gene (\( TNPI-C \)), and 3 highly polymorphic D-segment markers (\( D2S1471 \), \( D2S173 \), and \( D2S104 \)) located in the immediate vicinity of \( NRAMP1 \). The relative location of these markers in the chromosome region 2q35 is shown in figure 1.

![Figure 1](https://academic.oup.com/jid/article-abstract/181/1/302/894678)

For the intragenic \( NRAMP1 \) polymorphisms, choice of primers and polymerase chain reaction (PCR) were as described elsewhere [7, 16]. \( NRAMP1 \) restriction-fragment length polymorphisms were typed by PCR as described elsewhere [7]. \( D2S104 \) and \( D2S173 \) were typed by use of primers provided by Spurr et al. [17]. \( D2S1471 \) and \( TNPI-C \) were typed by use of primers and PCR as described by Copeman et al. [18] and Hoth and Engel [19], respectively.

**Statistical methods.** Comparisons of the quantitative Mitsuda reaction were made by variance analysis for categorical covariates (sex, ethnic origin, and clinical subtype) and regression analysis for age, taking into account the familial correlation of Mitsuda reaction measurements. All computations were done by use of the generalized linear model procedure with SAS software (SAS Institute, Cary, NC). The Bonferroni correction, accounting for multiple comparisons, was used for pairwise comparisons.

When considering the Mitsuda reaction as a quantitative trait, linkage analysis was done by use of 2 sib-pair methods. The first is the classical Haseman-Elston (HE) approach [20], which regresses the squared difference of the sib-pair phenotypes on the expected proportion of alleles shared identical by descent. Under the hypothesis of linkage, the regression coefficient is expected to be negative; that is, the lower the difference between the phenotypes of the sib-pair, the higher the proportion of alleles shared identical by descent. The test statistic was denoted as \( Z_{\text{HE}} \) and compared with a 1-sided standard normal deviate with respect to the large number of possible sib-pairs [21]. Computations for the HE method were done by means of the program SIBPAL in SAGE software [22]. However, in the presence of sibships with \( >2 \) sibs, the traditional HE method needs to decompose these sibships into all possible sib-pairs, which can raise some difficulties. Because our sample includes several large nuclear families, we also used the maximum-likelihood binomial (MLB) approach [23, 24], recently extended to the analysis of quantitative traits [25], which overcomes the problem of multiple sibs by considering the sibship as a whole.
idea is to introduce a latent binary variable \{0;1\} that captures the linkage information between the observed quantitative trait and the marker. In the present case, a value of 0 (or 1) for this binary variable can be understood as an “absolute” negative (or, respectively, positive) Mitsuda result. The method needs to specify the probability of the latent variable value (i.e., having a negative or a positive reaction) according to the observed quantitative result, and we used a standard cumulative normal distribution as proposed in [25]. As an example, the probability to have a value of 0 (negative Mitsuda result), given that the reaction is 0, 5, or 10 mm, is 0.93, 0.58, or 0.06, respectively. The likelihood of the observations is then written by use of binomial distributions of parental marker alleles among offspring according to the value of this unobserved binary variable, and depends on only 1 parameter, denoted as \(a\), which is the probability parameter of these binomial distributions. Under the null hypothesis of no linkage, \(H_0\), \(a = .5\) (parental allele transmission is independent of offspring phenotypes), whereas under the hypothesis of linkage, \(a > .5\) (sibs with closer phenotypes have more often received the same parental allele). The \(a\) parameter is estimated by maximum likelihood, and the test for linkage is a simple likelihood-ratio test assessing the departure of \(a\) from .5. For comparability with \(Z_{HE}\), this test was expressed in terms of a 1-sided standard normal deviate, denoted as \(Z_{MLBQ}\).

When considering the Mitsuda reaction as a 4-class categorical trait, the MLB method was the only usable approach. The difference with the quantitative case concerns the definition of the probability of the latent binary variable according to the observed categorical trait. We fixed the probability to have a 0 value (negative Mitsuda result) at 1, 0.75, 0.25, and 0 for phenotypes belonging to reaction categories of <3, 3–5, 5–10, and >10 mm, respectively. The rest of the method is identical to the quantitative case, in particular the linkage test, which is expressed in terms of a 1-sided standard normal statistic denoted as \(Z_{MLBC}\).

Large simulation studies under \(H_0\) of families with various sibship sizes showed that distributions of the MLB statistics were very close to the asymptotic ones, whereas inflation of type I errors was observed with the HE statistic [25]. Therefore, Monte Carlo methods were also used to compute \(P\) values by simulating 20,000 replicates of statistic values \((Z_{HE}, Z_{MLBQ}, Z_{MLBC})\) for our family sample under \(H_0\). With this method, the Monte Carlo \(P\) value (denoted as \(P_{MC}\)) is the proportion of replicates that provided a statistical value greater than or equal to the one observed in our data.

Results

The 20 nuclear study families included 118 sibs (59 with leprosy, 59 healthy) with available Mitsuda reaction and molecular NRAMP1 haplotype data. The number of families with 2, 3, 4, 5, 6, 7, 8, 9, 10, and 12 offspring was 2, 2, 2, 3, 5, 1, 1, 2, 1, and 1, respectively. All NRAMP1 haplotype segregation patterns were consistent with Mendelian transmission, and crossovers were checked as described in [8]. A heterozygosity of 100% was achieved in parents for the extended NRAMP1 haplotype, and an example of this haplotype observed in 2 families is presented in figure 2. The distribution of Mitsuda reactions among the 118 sibs is shown in figure 3A (quantitative) and 3B (categorical). The sib-sib correlation was estimated at 0.24 (\(P < .001\)) by use of REGRESS software [26], confirming the strong familial aggregation of Mitsuda reaction found in a Brazilian study [27]. Ages ranged from 5 to 40 years.
(mean, 23.2; SD, 7.4) and had no significant influence on the Mitsuda reaction. There was no significant difference of quantitative Mitsuda reaction according to sex or ethnic group, whereas a strong overall difference was observed among clinical subtypes \((P < 10^{-4})\), with mean Mitsuda reaction values of 6.42 mm \((n = 59)\), 7.50 mm \((n = 22)\), 6.21 mm \((n = 15)\), and 2.18 mm \((n = 22)\) among healthy, tuberculoid, indeterminate, and lepromatous subjects, respectively. Pairwise comparisons showed that lepromatous forms were clearly associated with a lower reaction to lepromin injection compared with indeterminate, tuberculoid, and unaffected subjects \((P < 10^{-4} \text{ for each comparison})\), whereas there was no significant difference between the 3 latter groups.

Linkage of the \(NRAMP1\) haplotype with the Mitsuda reaction was tested by use of the techniques summarized in Subjects and Methods, which provided very consistent results. When considering the Mitsuda reaction as a quantitative trait, the HE approach provided significant evidence for nonrandom segregation of \(NRAMP1\) haplotype in our sample \((Z_{\text{HE}} = -2.54; R_{\text{MC}} = .0104)\); however, support for genetic linkage was stronger with the MLB method \((Z_{\text{MLBO}} = 2.91; R_{\text{MC}} = .0017)\). Coding the Mitsuda reaction as a categorical trait provided a similar result \((Z_{\text{MLBC}} = 3.06; R_{\text{MC}} = .0010)\). As expected, the Monte Carlo \(P\) value was higher than the asymptotic \(P\) value \((P_{\alpha})\) for the HE method \((R_{\alpha} = .0055)\), whereas \(P\) values were very close with the MLB approach in both quantitative \((P_{\alpha} = .0018)\) and categorical \((P_{\alpha} = .0011)\) cases.

To further investigate the linkage between Mitsuda reaction and \(NRAMP1\), we performed 3 additional analyses of different subsamples by use of the categorical definition of the phenotype to avoid the problem of normality assumption. The results of these studies are shown in table 1. First, we used a linkage analysis focusing on sibs with extreme values (i.e., Mitsuda reactions <3 or \(\geq 10\) mm), because these values classically appear to be more reliable. This strategy is similar to linkage designs recently proposed by Risch and Zhang [28] for extremely discordant sib-pairs and by Gu et al. [29] for both discordant and concordant sib-pairs. Whereas the sample size strongly decreased (10 families, including 36 sibs, with extreme values), there was still significant evidence for linkage \((P = .0068)\). Interestingly, the estimation of the \(\alpha\) parameter (.77) was the same as the one observed in the whole sample. In this particular context of sibs with extreme values, there is a direct relationship between \(\alpha\) and the proportion of alleles shared by discordant sibs, which is equal to \(1 - 2\alpha (1 - \alpha)\) [23, 24]. Therefore, the proportion of parental alleles shared by sibs who have a Mitsuda reaction value <3 mm or by sibs who have a Mitsuda reaction value \(\geq 10\) mm is estimated at 0.646.

In a second step, we addressed the question of a possible genetic heterogeneity according to the ethnic origin of the families by performing separate linkage analyses among the Vietnamese and Chinese families, because such heterogeneity has been suggested in our previous linkage study between leprosy and \(NRAMP1\) [8]. The hypothesis of homogeneity was tested by minus twice the difference between the summed log likelihoods calculated in each subsample and the log likelihood obtained in the global sample and was not rejected \((\chi^2 = 0.49; 1 \text{ df}; \text{ not significant})\). As shown in table 1, there was significant linkage in the Vietnamese families, with an \(\alpha\) parameter estimated at .78 (compared with .77 in the whole sample), and only a trend in the much smaller Chinese subsample, but the estimation of \(\alpha\) was also close to .70.

Finally, we studied the presence of a possible confounding effect of the disease status in the present linkage results, because low Mitsuda-reaction values are associated with the lepromat-
tous subtype, and leprosy per se (i.e., all subtypes combined) was recently found to be linked to the \textit{NRAMP1} region [8]. Two separate analyses among healthy and affected subjects were conducted and showed evidence for linkage in both subsamples, with $P = .014$ and $P = .013$ for healthy and affected subjects, respectively. The estimation of the $\alpha$ parameter was also close in the 2 groups. These results support the view that the genetic linkage between Mitsuda reaction and \textit{NRAMP1} is independent of leprosy status.

**Discussion**

The present study shows a genetic linkage between the granulomatous response to lepromin and the \textit{NRAMP1} region among Vietnamese multiple leprosy-affected sibships, in which healthy sibs are very likely to have been exposed to \textit{M. leprae}. The Mitsuda reaction—\textit{NRAMP1} linkage appears to have a rather strong effect, as estimated by the high proportion of shared alleles (64.6%) in concordant-positive (Mitsuda reaction $\geq 10$ mm) and concordant-negative (Mitsuda reaction $< 3$ mm) sib pairs. This result obtained in large sibships also underlines the usefulness of the MLB approach, which avoids the decomposition into sib-pairs, can handle categorical phenotypes, allows the use of asymptotic distributions, and is found to be more powerful than the classical HE approach in the present analysis, as shown elsewhere by simulation studies [25].

Separate analyses among healthy and affected sibs show that linkage between the Mitsuda reaction and \textit{NRAMP1} is independent of leprosy status. An important question to which no definitive response could be brought by this relatively small sample is to what extent the linkage observed elsewhere between leprosy and \textit{NRAMP1} in the same families [8] can be accounted for by the present findings. Since positive or negative results of the Mitsuda reaction have been shown to be good predictors for resistance or susceptibility to lepromatous leprosy, and the proportion of borderline lepromatous and polar lepromatous cases reached 37% in our sample, it is possible that the present linkage can explain, at least in part, the leprosy-\textit{NRAMP1} linkage. However, other important macrophage functions that may be under \textit{NRAMP1} control, such as the bacteria-clearing capacity, have been shown to be better indicators of protective immunity against leprosy than is the Mitsuda reaction [30]. This indicates that \textit{NRAMP1} may play a role at different levels of the response against the leprosy bacillus. These hypotheses are under investigation in a larger sample of an ongoing study in which we will also test for linkage disequilibrium between the Mitsuda reaction and specific \textit{NRAMP1} alleles that could not be assessed in these 20 families.

Although we cannot rule out that another gene on chromosome region 2q35 in linkage with \textit{NRAMP1} is the underlying cause of the observed haplotype sharing, the results of our analyses suggest a regulatory role of \textit{NRAMP1} for the development of acquired antimycobacterial immune responses as determined by in vivo Mitsuda test activity. The Mitsuda reaction is a measure of the granuloma-forming capacity in response to \textit{M. leprae} antigen. In \textit{M. tuberculosis} infections, the proinflammatory cytokines interleukin-1 and tumor necrosis factor–$\alpha$ have been shown to be potent inducers of granuloma formation [31]. Interestingly, it has been well established that the \textit{Nramp1} allele in the mouse promotes significantly increased production of interleukin-1$\beta$ and tumor necrosis factor–$\alpha$, compared with that in \textit{Nramp1}${}^+$ animals [32, 33]. Moreover, increased production of both cytokines is common in developing Th1-type immune responses. For the mouse model, it is controversial to what extent \textit{Nramp1} regulates a selective difference in Th1 versus Th2 immunity [34–36]. However, multiple studies have shown a pronounced effect of \textit{Nramp1} alleles on the quality and quantity of the acquired immune response [37–41]. Enhanced cell-mediated immunity in response to \textit{M. bovis} (BCG) infection has been shown to be linked to increased secretion of plasminogen activator, a key event in granuloma formation [42]. Hence, our conclusion that \textit{NRAMP1} can play a role in the granuloma-forming capacity of humans is consistent with a large body of experimental evidence obtained in mouse models of mycobacterial infections.

It is not clear whether and to what extent modulations of murine acquired immune responses by \textit{Nramp1} reflect efficient antimycobacterial immunity. However, it has been shown that \textit{Nramp1} knockout mutants were fully able to control BCG infection in the phase of acquired immunity: homozygous \textit{Nramp1} knockout mice were able to clear BCG bacteria by 6 weeks after infection, and histologic examination of reticuloendothelial organs at 6 weeks after infection revealed the presence of well-formed granulomas in knockout mice, suggesting that, whereas the \textit{Nramp1}$^+$ allele promotes granuloma formation, it is not an essential element [43]. Moreover, no significant difference in in vivo antibody response to T cell–dependent antigens was found in \textit{Nramp1} (Bcg) congenic strains [44]. These results from congenic and gene deletion strains suggest that \textit{Nramp1} in the mouse plays its most important and crucial role in the innate phase of the macrophage-pathogen interaction. However, it is possible that the influence of \textit{Nramp1} on acquired immunity is dependent on the specific genetic background of the mouse strain used. Such a possibility would fit with the previous data that associated the \textit{Nramp1}$^+$ allele with enhanced immune responses.

It seems clear that \textit{Nramp1} in both mice and humans influences certain parameters of the antimycobacterial immune response. From a conceptual point of view, this is an important observation, because it expands the range of \textit{NRAMP1}-pathogen interaction from modulation of risk of infection to modulation of risk of disease development. Our results are consistent with the observation that \textit{NRAMP1} alleles may be associated with positive tuberculin skin test results [33] and the suggestion that \textit{NRAMP1} influences Th1/Th2 differentiation [33]. There is a major interest in identifying the genetic factors...
regulating the TH1/TH2 balance in response to foreign antigens, and a recent leprosy association study in India indicates that another genetic component influencing this TH1/TH2 shift may be the vitamin D receptor genotype [9]. The interplay between *NRAMP1* alleles and the quality and quantity of acquired antimycobacterial immunity likely holds the key to the understanding of how *NRAMP1* influences susceptibility to tuberculosis [10] and leprosy [8].

References


