A Major Susceptibility Locus on Chromosome 22q12 Plays a Critical Role in the Control of Kala-Azar

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Kala-azar (KA) is a life-threatening protozoal disease caused by *Leishmania* parasites (*L. donovani, L. chagasi,* and *L. infantum*). The disease, which is also called "visceral leishmaniasis," is prevalent in Africa, South America, Asia, and the Mediterranean basin. Epidemics occur periodically, killing a large number of infected individuals. Factors determining whether a patient remains asymptomatic or develops KA are still largely unknown. In a previous study that was performed during an outbreak of KA in a village on the Ethiopian-Sudanese border, we showed that KA was more frequent in certain families and ethnic groups, thereby suggesting that host genetic factors play an important role in the development of the disease. Here, we report the results of a genomewide linkage study performed on 63 Sudanese families selected from the most affected ethnic group and including 169 children with KA. Significant linkage (LOD score 3.50 [$P = 3 \times 10^{-5}$] in all patients; LOD score 3.90 [$P = 10^{-5}$] in patients who were affected early in the outbreak) was obtained with markers on chromosome 22q12. These results are the first evidence of a major genetic effect on the development of human KA. They may lead to identification of genes critical in the pathogenesis of this disease and to new therapeutic interventions against this parasite, which is developing resistance to available drugs.

Introduction

Visceral leishmaniasis is a disease caused by protozoa of the *Leishmania donovani* complex (*L. donovani, L. archibaldi,* and *L. infantum/chagasi*). Leishmania, which are introduced into their human hosts by sand flies, rapidly invade macrophages, where they multiply inside phagolysosomes. Human infections may be asymptomatic (i.e., subclinical) or may cause a severe visceral disease that is called "kala-azar" (KA) (Southgate and Manson-Bahr 1967; Badaro et al. 1986*a*, 1986*b*; Zijlstra et al. 1994). Clinical manifestations of KA include recurrent fever, hepatosplenomegaly, general lymphadenopathy, pancytopenia, and anemia; death occurs in the absence of appropriate chemotherapy (Pearson and Sousa 1996). Major KA outbreaks have occurred in India and the Sudan in recent years and have killed thousands of people (Seaman et al. 1996).

Factors that determine whether an infection remains asymptomatic or progresses to KA are largely unknown. Epidemiological studies have identified some environmental factors that affect exposure to *Leishmania* (Desjeux 1996, 2001). They have also indicated that the development of KA in infected individuals may depend on familial and ethnic factors (Zijlstra et al. 1994; Cabello et al. 1995; Ibrahim et al. 1999), suggesting that inherited factors may determine susceptibility to the disease in exposed populations. Genetics of infection by *Leishmania* has been studied in mice, and mutations in the *Nramp1* gene (Bradley et al. 1979; Vidal et al. 1995) and the *H-2* locus (Blackwell et al. 1980) were shown to affect the multiplication of *L. donovani*. The possibility that susceptibility to KA may be increased by mutations in certain genetic loci is also supported by experimental studies on *L. major* infections, which cause cutaneous leishmaniasis (CL) (Roberts et al. 1993; Demant et al. 1996; Beebe et al. 1997). Attempts to identify genes involved in susceptibility to leishmaniasis in humans have focused on candidate-gene testing (Blackwell 1996); associations have been reported between some *HLA* alleles (Barbier et al. 1987; Lara et al. 1991; Petzl-Erler et al. 1991) and *TNF* gene polymorphisms (Cabrera et al. 1995) and the risk of cutaneous and mucocutaneous leishmaniasis; however, the relevance of genetic susceptibility to KA has not yet been established.

Our group has undertaken a longitudinal study in a population during an outbreak of KA in a Sudanese village during 1995–2002. The seroepidemiological surveys, performed at different times, showed that almost

Table 1

Distribution of Families Acording to the Number of Sibs with KA

	NO. OF FAMILIES WITH				
FAMILY	Two Affected Sibs	Three Affected Sibs	Four Affected Sibs	Five Affected Sibs	TOTAL
First set (primary scan)	17	14	5	2	38
Second set	15	8	1	1	25
Total	32	22	6	3	63

NOTE.—All families were from the Aringa ethnic group and lived in the village of Barbar El Fugara (eastern Sudan).

all the population has been exposed to infection (Bucheton et al. 2003*b*) and that ∼25% of inhabitants have been affected by KA. Familial clustering of KA cases and differences in disease prevalence between ethnic groups indicated that this population was suitable for studies aimed at identifying susceptibility loci (Bucheton et al. 2002). A few candidate chromosomal regions were tested by linkage analysis on affected sib pairs, and suggestive linkage (LOD score 1.08; $P = .01$) was observed with a CA repeat in the *NRAMP1* gene promoter (Bucheton et al. 2003). We have now performed a genomewide search on this population and have identified a susceptibility locus for KA on chromosome 22q12 (LOD score 3.5; $P = 3 \times 10^{-5}$) and suggestive evidence of a second locus, on chromosome 2.

Material and Methods

Subjects and Families

Study subjects are from the village of Barbar El Fugara in eastern Sudan. The population of this village comprises mostly migrants from West Africa (members of the Hausa and Fellata ethnic groups) and the western Sudan/Chad area (members of the Aringa ethnic group), who started to settle as agricultural laborers in the 1940s. An outbreak of KA caused by *L. donovani, L. infantum,* and *L. archibaldi* (Pratlong et al. 2001) occurred in the village from 1995 to 2002 (El-Safi et al. 2002). All affected study subjects met the following three criteria for the diagnosis of KA: (i) presence of any two of the clinical symptoms (persistent fever, splenomegaly, general lymphadenopathy, loss of weight, and anemia); (ii) presence of parasites in lymph node aspirate; and (iii) positive response to treatment with Glucantime (Specia Rhone-Poulenc) or Pentostam (Wellcome Foundation). A seroepidemiological survey (Bucheton et al. 2003*b*) indicated that 90% of the village population harbored *Leishmania*-specific antibodies in the third year of the outbreak (1998). Twenty-eight percent of the village population developed KA during this outbreak, and most cases (90%) occurred in subjects ⩽30 years old (Bucheton et al. 2002).

To reduce genetic heterogeneity in the study sample,

only Aringa families (280 individuals from 63 families) were included. The 63 nuclear families selected for the study were not fully independent, since they were ascertained in 42 large pedigrees. The number of affected siblings who were genotyped is shown in table 1. The 63 families included 169 affected subjects (84 females; 85 males; age range 5–30 years [mean age 12.6 years]). Four subjects were ill in 1995, 29 in 1996, 73 in 1997, 33 in 1998, 24 in 1999, and 6 in 2000. Both parents were available in 39 families; only one parent was available in 20 families; and no parents were available in 4 families. When available, unaffected siblings were included, to infer the missing parents' genotypes ($N = 13$). Thirty-eight families were studied in the first stage of this genomewide linkage scan, and the 25 remaining families were included only in the second stage (table 1).

The study protocol was approved by both the federal and state Ministries of Health and by the Faculty of Medicine of Khartoum. Informed consent was obtained from the district authorities, from the village committee, from all participating adults, and from the parents of the children in the study. The regional health authorities and the village committee were informed on each of our visits of the purpose of the visit and the progress of the work.

Genotyping

The marker set used in the present study contained 367 microsatellite markers from the ABI Prism Linkage Mapping Set (MD10), version 2 (PE Biosystems), and 20 additional microsatellite markers (Dib et al. 1996). Three *NRAMP1* gene (2q35) polymorphisms—5′(CA)$_n$, 469+14G/C, and 1729+55del4—were also typed, as described elsewhere (Liu et al. 1995). Genotyping of microsatellite markers was performed as follows. DNA was extracted from blood samples according to a standard salting-out method. PCR assays with fluorescence-labeled primers were performed in 10-μl reactions containing genomic DNA (40 ng) and were set up with an automated pipetting station (Hamilton Microlab 4200). The DNA was amplified with each primer pair separately, pooled into panels, and run on ABI 377 sequencers (PE Biosystems), along with a size-standard ladder

(ROX or TAMRA; Applied Biosystems). The gels were analyzed using the Genescan analysis 3.1 and Genotyper 2.1 programs. Mendelian transmission of marker alleles was checked, and data from outlying individuals were either corrected or discarded before statistical analysis.

Statistical Analyses

Linkage analysis was performed using the maximum-likelihood binomial (MLB) model-free method (Abel et al. 1998). The approach is based on the binomial distribution of the number of affected siblings that have received a given parental allele, and it does not need to break down sibships with more than two affected into constitutive sibpairs. The likelihood of the data depends on only one parameter, which is denoted as "α" and is the probability parameter of this binomial distribution. There is a direct relationship between α and the proportion of alleles shared by the affected sibs, which is equal to $1-2\alpha(1-\alpha)$, regardless of the number of affected sibs (Abel et al. 1998). The α parameter is estimated by maximum-likelihood calculations, and the test for linkage is a simple likelihood-ratio test that assesses the departure of α from 0.5. The resulting statistic is asymptotically distributed as a 50:50 mixture of distributions with 0 and 1 df and can be expressed as a LOD score that has the same distribution as a LOD score based on a classical model that estimates the recombination fraction. Multipoint development of the MLB has been implemented (Abel and Muller-Myhsok 1998) in an extension of the Genehunter program (Kruglyak et al. 1996), and large simulation studies showed that the MLB statistic provided very consistent type I errors when asymptotic distributions were used, particularly in the analysis of families with more than two affected siblings (Abel and Muller-Myhsok 1998).

The locus-specific sibling recurrence-risk ratio (λ_s) was estimated by using the commonly used relationship between λ_s and the proportion of affected sibpairs sharing zero alleles (Risch 1990).

Results

KA affected only one-third of the village population, but >90% of the subjects were seropositive at the end of the outbreak; the transmission occurred in the village itself, most probably inside the huts (Lambert et al. 2002). Large differences in KA incidence were observed between ethnic groups, and incidence was highest in Aringa households (fig. 1*A*): 34.3% of the Aringa who were <30 years old developed the disease, compared with 10.1% and 14.6% for the Hausa and Fellata, respectively. As observed in other KA outbreaks, children and teenagers had the highest risk of developing the disease, particularly during the first years of the outbreak; KA was more frequent in adults toward the end of the out-
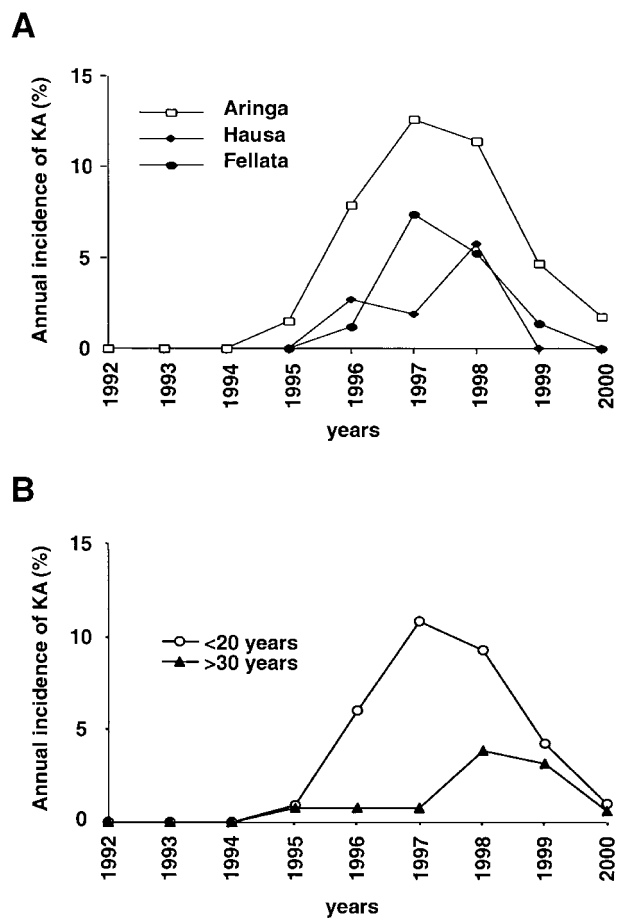
Figure 1 Annual incidence of KA during Barbar El Fugara village outbreak, according to ethnic group and age. *A,* Annual incidence of KA for subjects <30 years old, who represented 90% of the total KA cases. *B,* Annual incidence of KA for subjects <20 years old (*circles*) and for subjects >30 years old (*triangles*).

break (fig. 1*B*). Furthermore, most subjects who developed KA in 1999 or 2000 were seropositive for 1 or 2 years before the onset of disease (Bucheton et al. 2003*b*); this suggests long periods of asymptomatic carriage in these subjects.

A genomewide linkage study was performed on affected siblings, who were selected mainly from the most affected age groups (children and teenagers), and their parents. To reduce genetic heterogeneity of the study sample, only Aringa families were included. The linkage study was conducted in two steps. First, 38 families with multiple KA-affected siblings (table 1) were genotyped for 367 microsatellite markers covering the whole genome and providing a 10–15-cM primary genetic map. Figure 2 summarizes the results of the two-point LOD scores computed at each of the marker loci. Ten additional microsatellite markers were then genotyped on chromosomes 1, 2, 3, 7, 11, 14, and 22, close to markers with the highest LOD scores, to increase the informa-

tion content of these loci in the multipoint linkage analysis. Three regions—located on chromosomes 22q12, 2q24, and 2q35—yielded maximum multipoint LOD scores >0.83 ($P < .025$), with LOD scores of 2.9, 1.45, and 0.97, respectively (table 2). Two additional regions (14q13-14q23 and 2q27-3qter) provided lower LOD scores that were slightly below 0.83 (data not shown). All other regions provided LOD scores <0.73 ($P > .03$).

In the second step of the genetic analysis, the three regions that presented suggestive linkage with KA (LOD score $\geqslant 0.83$ [$P \leqslant .025$]) were further investigated by typing 25 additional families (table 2) and 13 additional markers: five microsatellites were typed at both the 22q12 and 2q23-2q24 loci, to provide a 2–3-cM map of these two regions, and three *NRAMP1* gene polymorphisms were typed at chromosome 2q35. In the latter analysis, LOD scores in region 22q12 increased to 3.50 ($P = 3 \times 10^{-5}$) at marker D22S280 (table 2; fig. 3). This locus is significant on a genomewide basis (Lander and Kruglyak 1995). Because our previous epidemiological studies suggested that KA occurring late in the outbreak developed against an immunological background different from that of the early cases (Bucheton et al. 2003*b*), the analysis was also performed on KA cases that occurred at the beginning of the outbreak (before 1999). In this analysis, 52 of the 63 families remained informative (with at least two affected children). An increased maximum multipoint LOD score of 3.90 ($P = 10^{-5}$) was obtained for a position 0.5 cM from the peak obtained when all KA cases were considered (fig. 3). The multipoint linkage analysis of the two other chromosomal regions (2q23-q24 and 2q35) yielded maximum multipoint LOD scores (1.53 and 0.79, respectively) similar to those in the first step (table 2). However, in an analysis performed on the 30 families displaying negative LOD scores at D22S280, the maximum multipoint LOD scores were increased to 2.29 at 2q23-q24 ($P = .0006$) and were moderately increased to 1.00 at 2q35 ($P = .015$) with *NRAMP1* intragenic polymorphisms. The chromosomal position of *NRAMP1* is 61 cM from the D2S141 marker that provides the maximum LOD score in the 2q23-q24 region.

Discussion

The present study shows a significant linkage of KA to chromosome 22q12 and provides suggestive linkage results at a second locus on chromosome 2q23-q24. This is the first report of a successful genome scan in human leishmaniais (CL or KA); it supports the view that susceptibility to KA is determined by host genetics, as was first indicated by animal studies (Blackwell 1996). Since the spread of KA is known to be markedly dependent on environmental conditions, the search for host genetic factors in KA was thought to be a difficult task. Several

factors may have contributed to the success of the present study: (i) it was performed in a population that experienced high transmission rates and the infection of a large majority of the population during the outbreak, thereby reducing the confounding effects of environmental factors (Bucheton et al. 2002); (ii) the KA phenotype was defined according to stringent criteria (in particular, the identification of the parasite on lymph node smears was an absolute requirement for patient inclusion); and (iii) the 5-year clinical and epidemiological study that preceded our genome scan detected several variables of heterogeneity in the population, including the ethnic origin of the subjects. We have been able to take these variables into account, thereby limiting sample heterogeneity. Nevertheless, an important source of heterogeneity that we could not control for was parasite diversity. During the epidemiological study, at least seven different zymodemes (characterized by isoenzyme electrophoresis) included in the three taxa *L. donovani, L. infantum,* and *L. archibaldi* were isolated from patients with KA (Pratlong et al. 2001). For this reason, we were concerned about the possibility that the search for susceptibility loci would be hindered by the complexity of the parasite population. This observation, as well as the failure of segregation analysis to detect a single major locus, led us to select the affected-sibling-pair strategy. The high virulence of *L. donovani* was also a major concern to us, since the possibility existed that very pathogenic parasites might have the capacity to overcome most genetic barriers. Finally, genetic control had never been analyzed in acute epidemic conditions, and there was concern that the genetic control might be more difficult to detect in such conditions than in endemic conditions and chronic illnesses. Fortunately, none of these factors hampered this genome scan or the demonstration of the genetic control. Genomewide linkage approaches have also been applied successfully in the study of genetic susceptibility to other infectious diseases. Levels of infection by *Schistosoma mansoni* are linked to chromosome 5q31-q33 (Marquet et al. 1996). In addition, two loci involved in susceptibility to leprosy were recently mapped: one locus, on chromosome 10p13, is involved in paucibacillary forms of the disease (Siddiqui et al. 2001), and another locus, on chromosome 6q25, controls leprosy itself (Mira et al. 2003). Taken together, these results demonstrate that genomewide approaches are valuable tools for the study of host genetics in complex infectious diseases. They also suggest that genetic susceptibility to infectious agents is less complex than was previously thought and that genetic variations at a single locus can significantly affect the susceptibility of the host.

The proportions of alleles shared by affected siblings in the region with the peak LOD score, 22q12, were 0.63 (all KA cases) and 0.66 (KA before 1999). The
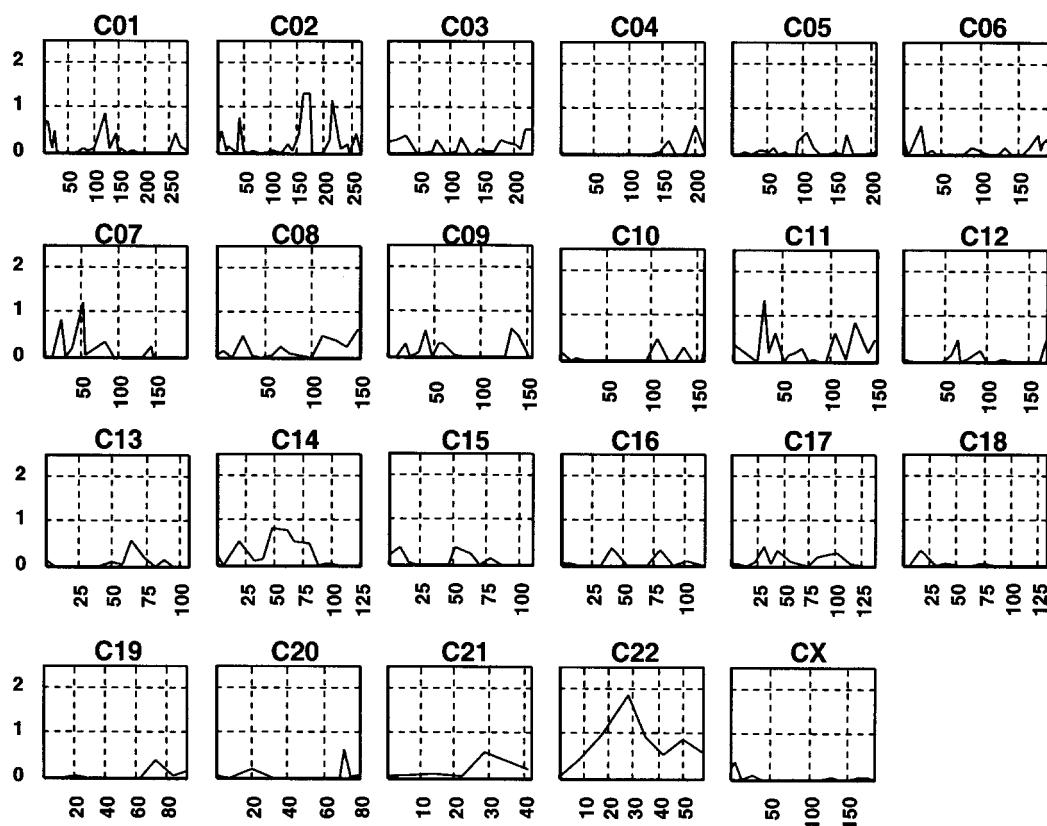
Figure 2 Primary genomewide linkage scan of 169 affected children belonging to 38 multicase nuclear families, using 377 microsatellite markers. The two-point LOD score is shown on the vertical axis and the distance (in cM) from the p terminus of the chromosome is on the horizontal axis.

locus-specific recurrence-risk ratios for siblings (λ_s) (Risch 1990) were estimated to be 1.83 (all KA) and 2.11 (KA before 1999). λ_s values for many infectious diseases are not expected to be very high, since λ_s cannot be greater than 1/disease prevalence (in this outbreak 1/0.3 = 3.33). For example, λ_s for a major locus of susceptibility to leprosy was 1.66 (Siddiqui et al. 2001). Although it is possible that the λ_s was overestimated because of an upward bias in the estimation of locus-specific effects from genomewide scans (Göring et al. 2001), the 22q12 locus identified here is likely to play an important role in the genetic control of KA in this population. The genotypic data from the 30 patients who developed KA late in the outbreak (in 1999 and 2000) contributed little to the LOD score that mapped a KA-susceptibility gene on chromosome 22q12. This indicates that the control of KA in subjects affected early in the epidemic may be different from that in subjects affected later. This view is supported by the observation that subjects affected late in the epidemic, unlike those affected earlier, were found to control infection for several years before the onset of clinical disease (Bucheton et al. 2003*b*). Evidence of the genetic contribution to

host susceptibility to KA will be further increased if the second locus can be confirmed, since our data suggest a genetic heterogeneity, with a major locus on chromosome 22q12 and another on chromosome 2q23-q24 (LOD score 2.29) in families with negative LOD scores at the 22q12 locus. Suggestive linkage (LOD score 1.00) was also seen with *NRAMP1* intragenic markers (2q35), which confirms our previous results obtained with a smaller sample size (Bucheton et al. 2003). Thus, our work suggests that *NRAMP1*, unlike its mouse homolog, plays only a minor role in determining susceptibility to KA. However, one should note that *Nramp1* was shown to influence early control of parasite loads in the spleens of mice experimentally infected with *L. donovani* (Bradley et al. 1979), a phenotype that may be different from the clinical phenotype studied here. Furthermore, susceptibility was shown to be caused by the *Nramp1*[Asp169] functional null allele, and, although allelic forms of the human *NRAMP1* gene have been shown to be associated with increased susceptibility to various infectious or autoimmune diseases (Blackwell et al. 2003), there is no evidence that such null alleles of the *NRAMP1* gene exist in human populations ex-

Table 2

Results of the Linkage Analysis Performed on Families with Sibs Affected by KA

CHROMOSOMAL REGION AND MARKER	LOD SCORE (% INFORMATION CONTENT) IN					
	Primary Scan		Refined Map		Conditional Analysis	
	Single Point	Multipoint	Single Point	Multipoint	Single Point	Multipoint
22q12:						
D22S315	.97 (61)	1.54 (80)	1.07 (64)	1.77 (84)	...	...
D22S1167	...	...	.56 (38)	2.54 (81)	...	...
D22S1163	...	...	.97 (56)	2.44 (83)	...	...
D22S280	1.84 (59)	2.90 (82)	1.66 (56)	3.50 (86)	...	...
D22S424	...	...	.27 (34)	2.40 (86)	...	...
D22S277	...	...	.74 (72)	1.64 (92)	...	...
D22S283	.88 (87)	1.09 (96)	1.67 (84)	1.67 (95)	...	...
2q23-2q24:						
D2S151	.40 (73)	.44 (84)	.67 (66)	.72 (85)	2.19 (70)	2.27 (87)
D2S2275	...	...	1.89 (47)	1.00 (87)	2.85 (42)	2.21 (88)
D2S2299	...	...	1.23 (57)	.99 (89)	1.62 (63)	1.75 (90)
D2S141	...	...	2.14 (66)	.86 (93)	3.74 (72)	2.29 (93)
D2S142	1.28 (71)	1.45 (83)	2.34 (70)	1.23 (93)	3.84 (71)	2.27 (94)
D2S284	...	...	.10 (50)	.93 (88)	.39 (50)	2.06 (87)
D2S156	...	...	.45 (65)	.95 (87)	1.99 (69)	2.07 (87)
D2S2330	1.27 (63)	1.22 (82)	2.01 (58)	1.53 (81)	1.78 (62)	2.26 (81)
2q35:						
D2S2382	1.15 (68)	.97 (83)	.84 (69)	.79 (84)	1.70 (68)	.95 (83)
$5'(CA)_n$[a]	...	...	.60 (30)	.77 (84)	.17 (32)	.98 (58)
469+14G/C[a]	...	...	.21 (23)	.73 (84)	.38 (23)	1.00 (84)
1729+55 del4[a]	...	...	.38 (22)	.50 (86)	.37 (24)	.71 (87)
D2S1471	.25 (64)	.31 (90)	.07 (69)	.18 (90)	.14 (70)	.50 (90)

NOTE.—Linkage results are shown for markers of three chromosomal regions (22q12-q13, 2q23-q24, and 2q35) with LOD scores >0.83 ($P < .025$) in the primary screen of the genome conducted on a set of 38 families and for the whole family set (63 families). An analysis was performed on the 30 families displaying negative LOD scores at D22S280. Linkage analyses were performed using the MLB model-free method implemented in an extension of the Genehunter program.

[a] *NRAMP1* intragenic polymorphisms.

posed to a wide range of intracellular pathogens. It is noteworthy that a study of mice from wild natural populations also failed to identify the null allele (Blackwell 1983).

The knowledge that KA is, in part, determined by a major locus and possibly by a second locus is the first step toward the identification of the disease susceptibility genes and the characterization of the critical steps in the pathological process involved in this lethal disease. The IL-2 receptor β chain gene (*IL2RB*), which is located in the 22q12 chromosomal region close to D22S280 microsatellite marker, is a good candidate for the control of KA. IL-2 is critical for T-cell proliferation and differentiation. T-cell responses are strongly suppressed in patients with KA (Carvalho et al. 1981, 1985), and high levels of circulating soluble IL-2 receptor have been observed during the acute phase of the disease (Barral-Netto et al. 1991). Studies of other protozoa, such as *Trypanosoma cruzi,* also support the hypothesis that some parasites evade the immune system by acting on the IL-2 pathway (Majumder and Kierszenbaum 1996). In this regard, it is interesting to note that two other genes—N-myc interactor (*NMI* [MIM 603525]) and signal transducing adapter molecule 2 (*STAM2* [MIM 606244]), both located in the 2q22-q23 region—code for proteins involved in the IL-2 signaling-transduction pathway.

These results raise the possibility that host susceptibility alleles play a critical role in the control of disease outbreaks. This view is supported by the marked differences in susceptibility to KA among the three ethnic groups of the village, differences that could not be ascribed by epidemiological factors (Bucheton et al. 2002). It would be premature to draw conclusions at this stage of the study; however, the identification of the susceptibility alleles at the 22q12 locus and at other chromosomal locations should allow studies that will lead to stronger conclusions about the role of susceptibility alleles in disease outbreaks.
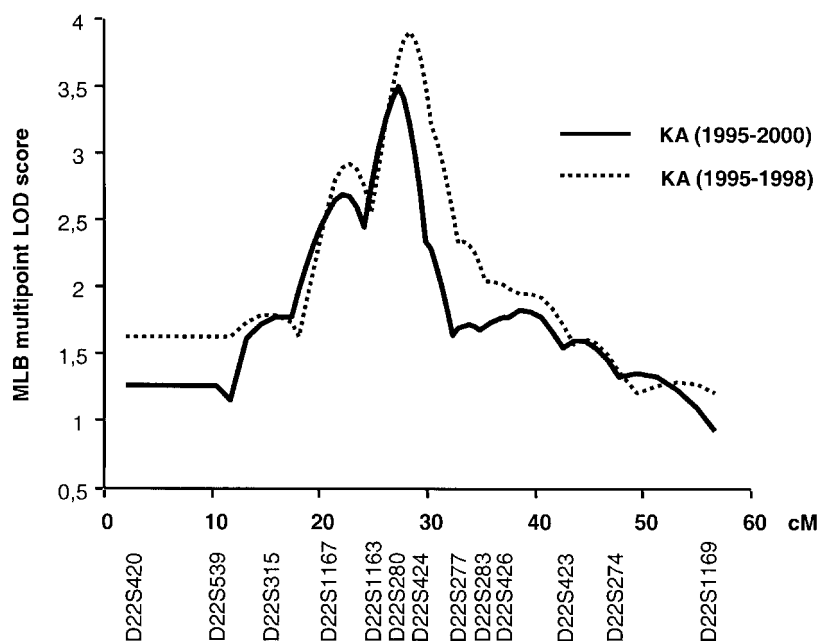
Figure 3   Multipoint analysis localizing a major KA-susceptibility locus on chromosome 22. Results of the multipoint linkage analysis performed on the whole family set with chromosome 22 microsatellite markers. Multipoint LOD scores were computed on all KA cases (1995–2000; 63 families) (*solid line*). Multipoint LOD scores were also computed on KA cases that occurred before 1999 (*dashed line*). Fifty-two families remained informative in this analysis (with the following distribution: 33, 14, 3, and 2 families with 2, 3, 4, and 5 affected children, respectively).

Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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