

Atopy in children in relation to BCG vaccination and genetic polymorphisms at SLC11A1 (formerly NRAMP1) and D2S1471

JS Alm^{1,4}, CB Sanjeevi², EN Miller³, P Dabadghao², G Lilja⁴, G Pershagen⁵, JM Blackwell³ and A Scheynius¹

¹Department of Medicine, Unit of Clinical Allergy Research, Karolinska Hospital and Institutet, Stockholm, Sweden; ²Department of Molecular Medicine, CMM, Karolinska Hospital, Stockholm, Sweden; ³Cambridge Institute for Medical Research (CIMR), University of Cambridge School of Clinical Medicine, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Cambridge CB2 2XY, UK; ⁴Sachs' Children's Clinic, Söder Hospital, Stockholm, Sweden; ⁵Division of Environmental Epidemiology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden

*The influence of the SLC11A1 (formerly NRAMP1) gene on the association between BCG vaccination and atopy in children was studied in 179 children, BCG vaccinated in infancy, and 307 children without BCG vaccination, all 3 to 8 years of age and with atopic heredity. DNAs were genotyped for a functional repeat polymorphism (designated GT_n) in the promoter of SLC11A1 and a linked microsatellite D2S1471. Associations between genotype, atopic symptoms and allergen-specific IgE-antibodies in relation to the BCG status of the children were assessed. Atopy in relation to SLC11A1 GT_n-alleles was similarly distributed between the two groups. In BCG vaccinated children, genotype associations were observed for D2S1471 and atopy, with carriage of allele 5 conferring increased risk of atopy (odds ratio (OR) 2.6; 95% confidence interval (CI) 1.3–5.5; *P* = 0.01), and particularly IgE responses to airborne allergens (OR = 4.3; 95% CI 1.7–10.7; *P* = 0.002). No linkage disequilibrium was found between the SLC11A1 GT_n repeat and the microsatellite D2S1471, and therefore no haplotype associations were observed for atopy in BCG- or non-BCG vaccinated children. Stratification by BCG vaccination unmasked a potential genetic risk factor for atopy in the region of the SLC11A1 locus, and point to the importance of genotype by environment interactions in determining disease susceptibility.*

Genes and Immunity (2002) 3, 71–77. DOI: 10.1038/sj/gene/6363834

Keywords: atopy; BCG; children; D2S1471; NRAMP1; SLC11A1

Introduction

Atopic diseases, consisting of food allergy, atopic dermatitis, allergic asthma and rhinoconjunctivitis, have shown a considerably high prevalence among children in many countries, especially those with a 'Western lifestyle'.¹ Genetic, but also environmental factors are important in the aetiology of atopy.² Following exposure to an allergen, an antigen-presenting cell processes the allergen to peptides, which becomes associated with major histocompatibility complex (MHC) molecules. On the cell surface MHC class II-molecule interaction occurs with CD4⁺ T-cells via specific receptors. The activated CD4⁺ cell can

differentiate into T-helper1 (Th1)-like T-lymphocytes, which mainly produce interferon- γ (IFN- γ), a principal regulator of cellular immunity, or into Th2-like T-cells producing interleukine-4 (IL-4) and other cytokines of importance for IgE synthesis.³ A Th2-like immune response dominates in atopic individuals, an imbalance which is thought to be a result of both constitutional and environmental impacts.³ According to the hygiene hypothesis,⁴ external exposures like changes in the panorama of infectious diseases could constitute a possible cause of the increased prevalence in atopic diseases. For example, an inverse relation between atopy and orofaecal transmitted diseases such as hepatitis A and toxoplasma has been observed.⁵ An inverse correlation between size of tuberculin skin indurations and atopy has also been observed in Bacillus Calmette-Guérin (BCG)-vaccinated school children in Japan.⁶ Animal studies support an association between response to mycobacteria and allergic responses.^{7,8} These data are consistent with BCG's action as a potent adjuvant for induction of cell-mediated immunity, with IFN- γ as one of the major cytokines induced.⁹ IFN- γ is associated with a Th1-like immune response and counteracts production of IgE antibodies.³ Since ability to mount an immune response to mycobacterial antigens is highly heritable,¹⁰ a genetic

Correspondence: Dr JS Alm, Sachs' Children's Clinic, Söder Hospital, S-118 83 Stockholm, Sweden. E-mail: Johan.Alm@sos.ki.se

This study was financially supported by the Swedish Medical Research Council (grants no 16x-7924, 72P-13149), the Swedish Foundation for Health Care Sciences and Allergy Research, the Swedish Asthma and Allergy Association, the Swedish Council for Work Life Research, Consul Th C Berg's Foundation, the Samariten Foundation, the Hesselman's Foundation, and the Karolinska Institutet. The work carried out in Cambridge was supported by grants from The Wellcome Trust.

Received 15 August 2001; revised 1 November 2001; accepted 5 November 2001

contribution to the association between tuberculin reactivity and atopy is also plausible. Whilst the association between BCG vaccination and atopy remains controversial, with further epidemiological studies reporting both a strong inverse correlation¹¹ and others as no effect,^{12–14} no studies have yet introduced genetics as a risk factor regulating this association.

One interesting candidate gene already known to control immune response to mycobacteria is the natural resistance associated macrophage protein 1 (NRAMP1), now renamed SLC11A1. In mice, *Slc11a1* controls response to *Mycobacterium bovis* BCG, as well as to a range of other intracellular macrophage pathogens (reviewed in Blackwell *et al*¹⁵). In man, SLC11A1 has been shown to be linked or associated with both infectious^{16–23} and autoimmune^{24–30} diseases. The protein encoded by *Slc11a1*/SLC11A1 localizes to membranes of late endosome/lysosomes,^{31,32} and is a proton/divalent cation antiporter.³³ Whilst divalent cation transport may directly affect antimicrobial activity through contribution of Fe²⁺ to the Fenton Reaction,³⁴ the central role of divalent cations in many cellular functions provides the basis to multiple pleiotropic effects of the SLC11A1 gene (reviewed in Blackwell *et al*¹⁵). One such pleiotropic effect is enhanced antigen processing for presentation to T cells,³⁵ which may reflect metal ion requirement for metalloprotease activity and/or endosomal fusion events. This effect on antigen processing is compounded by *Slc11a1*'s influence on molecules regulating (TNF- α , IL-1 β) or directly involved in (MHC class II) antigen presentation.¹⁵ Hence, in mice vaccinated with attenuated *Salmonella typhimurium* engineered to express tetanus toxoid fragment C, polarised Th1 *vs* Th2 responses are observed in *Slc11a1* congenic wild-type *vs* mutant mice.³⁶ This Th1:Th2 bias is also seen in mycobacterial infection in *Slc11a1* congenic mice.³⁷ In Malawi, significant SLC11A1 haplotype associations with IFN- γ responses to a range of mycobacterial antigens from *M. tuberculosis*, BCG or environmental mycobacteria have been observed (PEM Fine, JM Blackwell and others, unpublished data) consistent with the murine Th1:Th2 bias. These results suggest that polymorphism at SLC11A1 influences immune response to 'priming/vaccinating' exposures to mycobacteria. This is interesting in that BCG's effectiveness in protecting against leprosy in the same Malawian population is also associated with polymorphism at SLC11A1 (AV Hill and PEM Fine, personal communication). No SLC11A1 association with leprosy is observed in non-BCG vaccinated subjects. Polymorphism at SLC11A1 is also associated with Mitsuda-type skin test reactivity to leprosy antigens in Vietnam.³⁸

Given the potential impact of SLC11A1 in determining the Th1:Th2 bias in response to environmental or vaccination exposure to mycobacterial antigens, we decided to examine SLC11A1 as a candidate gene for atopy, comparing BCG *vs* non-BCG vaccinated children in an allelic association study. DNA from atopic and non-atopic children with atopic heredity was analysed in relation to their BCG vaccination status and polymorphisms at SLC11A1 and the tightly linked microsatellite D2S1471.

Results

Following randomised exclusion of a sibling in order to eliminate clustering within families, and of children with-

out parental consent for genetic analysis, DNA was available for 179 of 216 BCG vaccinated children and for 307 of 358 non-BCG vaccinated children from a previous study.¹² SLC11A1 could be typed in 175 (98%) and 297 (97%), respectively; D2S1471 in 165 (92%) and 297 (97%), respectively. Geometric mean age by vaccination in the BCG group was 4 days. Atopy, defined as positive serological test and/or skin-prick test to any of the selected allergens, occurred in 54 (30%) children in the BCG group, compared with 80 (26%) in the non-BCG group. Gender was similarly distributed between the two groups, 47% and 49% females, respectively. By likelihood ratio test no effect modulation of age by BCG vaccination on atopy was found (data not shown).

SLC11A1 GT_n and atopy

Distribution of SLC11A1 genotypes according to atopic status in BCG vaccinated and non-BCG vaccinated children are shown in Table 1. No significant differences in genotype frequencies were observed between any of the four groups of children, ie BCG vaccinated/non-BCG vaccinated and with/without atopy. Combined allele frequencies (0.01, 0.27 and 0.72 for alleles 1, 2 and 3 respectively) were similar to those reported in previous studies of European populations.^{27,28} Using logistic regression no statistically significant interaction could be shown between BCG vaccination and the SLC11A1 GT_n in relation to atopy. Symptoms and history congruent with atopy were similarly distributed between the two groups in relation to SLC11A1 alleles, also assessed by logistic regression (data not shown). Age by BCG vaccination, gender, parental place of birth, or age at sampling had no influence on the effect of BCG on the occurrence of atopy in relation to SLC11A1 alleles (data not shown).

D2S1471 and atopy

Seventeen alleles of D2S1471 were identified in this study population. Only alleles 5 and 11 occurred at frequencies >0.1. The likelihood ratio test showed no overall allelic associations between the four groups of children. However, within the BCG vaccinated group an overall χ^2 statistic of 23.59 with 15 degrees of freedom achieved a *P*-value of 0.07 for association between D2S1471 alleles and atopy. No equivalent association was seen in the non-BCG-vaccinated group, or for the total sample without stratification for BCG vaccination status. Analysis of genotype frequencies for the common alleles (Table 2) showed that the association in BCG-vaccinated children appeared to be due to differences between non-atopic and atopic children in frequencies of genotypes carrying one or two copies of allele 5 compared with all other genotype combinations (χ^2 5.72; 2 d.f. *P* = 0.06). Logistic regression analyses, including adjustment for potential confounders, showed that carriage of at least one copy of allele 5 conferred increased risk of atopy in BCG vaccinated children (odds ratio (OR) 2.6; 95% confidence interval (CI) 1.3–5.5; *P* = 0.01) (Table 2). In contrast, allele 11 showed no significant genotypic or allelic associations with atopy in the BCG vaccinated group (data not shown). No associations were seen with rare (<0.1) alleles. The risk attributed to allele 5 in the BCG vaccinated group was particularly elevated for IgE responses to airborne allergens (OR 4.3; 95% CI 1.7–10.7; *P* = 0.002) (Table 2). Although no overall allelic or genotype associations were found in the non-BCG-vaccinated group,

Table 1 Distribution of SLC11A1 genotypes in BCG vaccinated or non-vaccinated children in relation to atopy and sub grouped by IgE responses to food or air-borne allergens

	BCG vaccinated (n = 175)				Non-BCG vaccinated (n = 297)			
	Atopy ^a							
	Neg		Pos		Neg		Pos	
SLC11A1	n = 122	(%)	n = 53	(%)	n = 223	(%)	n = 74	(%)
1;2	0		0		0		1	(1.4)
1;3	3	(2.5)	1	(1.9)	4	(1.8)	2	(2.7)
2;2	19	(15.6)	8	(15.1)	25	(11.2)	6	(8.1)
2;3	38	(31.1)	14	(26.4)	67	(30.0)	21	(28.4)
3;3	62	(50.8)	30	(56.6)	127	(57.0)	44	(59.4)
	Food ^b							
	Neg		Pos		Neg		Pos	
SLC11A1	n = 139	(%)	n = 36	(%)	n = 252	(%)	n = 45	(%)
1;2	0		0		0		1	(2.2)
1;3	3	(2.1)	1	(2.8)	6	(2.4)	0	
2;2	21	(15.1)	6	(16.7)	28	(11.1)	3	(6.7)
2;3	40	(28.8)	12	(33.3)	76	(30.2)	12	(26.7)
3;3	75	(54.0)	17	(47.2)	142	(56.3)	29	(64.4)
	Air borne ^c							
	Neg		Pos		Neg		Pos	
SLC11A1	n = 137	(%)	n = 38	(%)	n = 252	(%)	n = 45	(%)
1;2	0		0		0		1	(2.2)
1;3	3	(2.2)	1	(2.6)	4	(1.6)	2	(4.4)
2;2	21	(15.3)	6	(15.8)	27	(10.7)	4	(8.9)
2;3	43	(31.4)	9	(23.7)	75	(29.8)	13	(28.9)
3;3	70	(51.1)	22	(57.9)	146	(57.9)	25	(55.6)

^aDefined as positive serological test and/or skin-prick test to any of the selected allergens. ^bPositive IgE response to food allergens. ^cPositive IgE response to air-borne allergens.

stratification by type of allergy showed that IgE response to food allergens appeared less common among children with allele 5 within the non-vaccinated group. Symptoms or history congruent with atopy were similarly distributed between the two groups, also in relation to presence of allele 5 (data not shown). Age by BCG vaccination, gender, parental place of birth, or age at sampling had no strong influence, if any, on the effect of BCG on the occurrence of atopy in relation to D2S1471 alleles (data not shown).

Linkage disequilibrium and haplotyping

Logistic regression analyses did not indicate any interaction between SLC11A1 GT_n alleles and D2S1471 alleles. This is consistent with values obtained for W_n in the non-atopic children (W_n 0.23; $P < 0.01$), indicating that there is no linkage disequilibrium between alleles at the 5' promoter region SLC11A1 GT_n polymorphism and the 3' D2S1471 microsatellite in this population. In accordance with this observation, no overall associations were observed for SLC11A1 GT_n – D2S1471 haplotypes for any comparison between non-atopic and atopic children whether BCG vaccinated or not (data not shown).

Discussion

Previous analysis of risk factors for atopy in this cohort of children had concluded that BCG vaccination did not confer pre-school age protection against atopy in these children of atopic heredity.¹² The aim of the current study was to determine whether taking account of SLC11A1 genotype as a genetic risk factor could uncover a previously masked association between response to BCG vaccination and susceptibility to atopy. No association was observed between atopy and the functional GT_n SLC11A1 promoter region polymorphism in either BCG vaccinated or non-vaccinated children, indicating that this polymorphism is not itself a risk factor for disease. Interestingly, however, allelic and genotypic associations were observed between atopy and the D2S1471 microsatellite lying ~68 kb 3' of SLC11A1 within the group of BCG vaccinated children, as compared with the non-BCG vaccinated children. D2S1471 lies within the VIL1 gene, with genes encoding the two functional interleukin 8 receptors (IL8RA, IL8RB)³⁹ and a pseudogene lying between SLC11A1 and VIL1. Hence, we cannot discount the possibility that polymorphism at the IL8R genes in

Table 2 Distribution of D2S1471 genotypes in BCG vaccinated or non-vaccinated children in relation to atopy and sub grouped by IgE responses to food or air-borne allergens

	BCG vaccinated (n = 165)				Non-BCG vaccinated (n = 297)			
	Atopy ^a							
	Neg		Pos		Neg		Pos	
D2S1471	n = 112	(%)	n = 53	(%)	n = 217	(%)	n = 80	(%)
5;5	17	(15.2)	10	(18.9)	31	(14.2)	7	(8.9)
5;non-5	46	(41.1)	30	(56.6)	101	(46.3)	34	(43.0)
non-5;non-5	49	(43.7)	13	(24.5)	86	(39.5)	38	(48.1)
OR; 95% CI; P	*2.6; 1.3–5.5; 0.01				*0.64; 0.38–1.1; 0.1			
	Food ^b							
	Neg		Pos		Neg		Pos	
D2S1471	n = 129	(%)	n = 36	(%)	n = 247	(%)	n = 50	(%)
5;5	20	(15.5)	7	(19.4)	34	(13.8)	4	(8.0)
5;non-5	57	(44.2)	19	(52.8)	117	(47.4)	18	(36.0)
non-5;non-5	52	(40.3)	10	(27.8)	96	(38.8)	28	(56.0)
OR; 95% CI; P	*1.7; 0.78–3.9; 0.2				*0.49; 0.27–0.91; 0.03			
	Air borne ^c							
	Neg		Pos		Neg		Pos	
D2S1471	n = 127	(%)	n = 38	(%)	n = 248	(%)	n = 49	(%)
5;5	19	(15.0)	8	(21.1)	34	(13.7)	4	(8.2)
5;non-5	53	(41.7)	23	(60.5)	110	(44.4)	25	(51.0)
non-5;non-5	55	(43.3)	7	(18.4)	104	(41.9)	20	(40.8)
OR; 95% CI; P	*4.3; 1.7–10.7; 0.002				*0.97; 0.51–1.8; 0.9			

^aDefined as positive serological test and/or skin-prick test to any of the selected allergens. ^bPositive IgE response to food allergens. ^cPositive IgE response to air-borne allergens.

*5;5 + 5;non-5 alleles compared to non-5;non-5 alleles by logistic regression, adjustment for age at sampling and gender.

linkage disequilibrium with allele 5 of D2S1471 contribute to the increased risk of atopy observed in BCG vaccinated children. However, given that a strong association is only observed in the BCG vaccinated group, and that SLC11A1 is known to regulate immune response to mycobacterial infection and vaccination, it seems more likely that the association observed is due to linkage disequilibrium between D2S1471 and 3' haplotypes in SLC11A1. This is consistent with previous work⁴⁰ in a Vietnamese population, where no linkage disequilibrium was observed between 5' and 3' polymorphic markers within the SLC11A1 locus, but evidence was obtained for linkage disequilibrium between 3' SLC11A1 markers and D2S1471. Also with the demonstration that both 5' and 3' polymorphisms at SLC11A1 are independently associated with susceptibility to tuberculosis in the Gambia,¹⁷ indicating that there are likely to be as yet unidentified functional polymorphisms at the 3' end of the SLC11A1 gene. Thus, overall our data demonstrate that stratification by BCG vaccination has unmasked a potential genetic risk factor for atopy in the region of the SLC11A1 locus on human chromosome 2q35, and point to the importance of genotype by environment interactions in determining disease susceptibility.

SLC11A1 is known to control susceptibility to a range of infectious^{16–23} and autoimmune^{24–30} diseases in man. Whilst susceptibility to infection may in part be deter-

ined by the direct influence of SLC11A1 on antimicrobial activity of macrophages, it seems likely that its pleiotropic effects in regulating Th1:Th2 balance in the immune response^{36,37} will contribute to both autoimmune and infectious disease susceptibility. Indeed, one of the current perplexities in SLC11A1 research has been the clear association between both 5' and 3' haplotypes and global susceptibility to pulmonary tuberculosis in man,^{17–20,22,23} but lack of any effect of SLC11A1 on primary infection with *M. tuberculosis*^{41–43} in mice. One clear difference is that humans, especially in Africa, are repeatedly exposed to environmental mycobacteria prior to infection with *M. tuberculosis*. The demonstration (PEM Fine, JM Blackwell and others, unpublished data) that polymorphism at SLC11A1 influences immune response to 'priming/vaccinating' exposures to mycobacteria in Malawi provides some explanation for this. It is of interest too that it was a 3' haplotype of SLC11A1 in linkage disequilibrium with D2S1471 that showed significant association with Th1 interferon- γ responses to *M. tuberculosis*, BCG and a range of environmental mycobacteria including *M. fortuitum*. These data suggest that SLC11A1-regulated responses to environmental mycobacteria could also influence progression to atopic disease in places like Africa where environmental exposure is high. Further studies have shown, however, that even on this background of high environmental exposure to myco-

bacteria, BCG's effectiveness in protecting against leprosy is also associated with polymorphism at SLC11A1 (AV Hill and PEM Fine, personal communication). As with the atopy study, no SLC11A1 association with leprosy was observed in non-BCG vaccinated subjects.

It was of interest in our study that the association we observed between D2S1471 and atopy within the BCG vaccinated children was with IgE response to airborne but not food allergens. These results suggest that SLC11A1 genotype can influence bias in the Th1:Th2 response to non-mycobacterial antigens and allergens in BCG vaccinated individuals, but it seems likely that the route of allergen entry may be important in determining this response. Although SLC11A1 has been associated with gut immune responses by virtue of its linkage to Crohn's disease,²⁵ nothing is known about its influence on Th1:Th2 bias in the gut, or whether it could influence development of tolerance to food allergens.

In conclusion, the results obtained here suggest that polymorphism(s) in the genomic region of SLC11A1 are associated with risk of atopy in BCG vaccinated children. Further work is required to pinpoint the polymorphism(s) involved, and to confirm the underlying immunological mechanism by which immune response to BCG vaccination or environmental mycobacteria can influence atopy. Overall our results support the hypothesis that gene/environment interactions are likely to be important in determining susceptibility to complex disorders like atopy and autoimmunity.

Methods

Subjects

In a retrospective cohort study described elsewhere¹² 216 children, 3–8 years of age who received BCG vaccination when they were younger than 6 months of age, were identified at their Child Health Centres. They were born in Stockholm, Sweden, between 1989 and 1992 and compared with 358 age-matched children living in the same area, who had not been BCG vaccinated. To increase the efficiency of the study, only children whose parents had history of atopy were included. The parents were all of Nordic ethnicity (Swedish born (95%), Finnish born (4.0%), Danish born (0.5%) or Norwegian born (0.5%). A physician assessed clinical symptoms or history congruent with atopic disease. Atopy was also assessed by analysis of allergen specific IgE antibodies in sera (Pharmacia Diagnostics AB, Uppsala, Sweden) using six common food allergens (hen's-egg white, codfish, cow's milk, peanut, soy bean and wheat flour (fx5)) and 11 airborne allergens (cladosporium, *D. farinae*, *D. pteronyssinus*, cat, dog, horse, birch, timothy grass, mugwort, olive and *Parietaria judaica* (Phadiatop®)). In addition, skin prick tests (SPT) (ALK, Copenhagen, Denmark) were performed against 13 common allergens: birch, cat, cladosporium, *Dermatophagoides pteronyssinus*, dog, horse, mugwort, timothy grass (Soluprick, 10 HEP, ALK) and hen's egg white (Soluprick, w/v 1/100), codfish (Soluprick, w/v 1/20), cow's milk (3% fat, standard foodstuff), peanut (Soluprick, w/v 1/20) and soy bean (Soja Semp, Semper AB, Stockholm, Sweden). The same batches of individual extracts were used and the SPTs were performed by the same well-trained nurse throughout the study, who also did not know if the child was

BCG-vaccinated or not. Children with a positive serological test and/or at least one positive SPT (≥ 3 mm in diameter and $\geq$ the histamine-induced weal after 15 min) to any of the selected allergens were considered to be atopic. The term atopy here exclusively refers to such positive tests. The BCG used in all vaccinated children was 0.05 ml attenuated Kb 1331 strain of *Mycobacterium bovis* (Statens seruminstitut, Copenhagen, Denmark), and a scar on the vaccination site was confirmed in the clinical assessment. Since BCG vaccination is generally associated with Th1:Th2 immune balance, the prevalence of Th1-type response associated diseases³ was assessed in parallel. Only one of the 486 children (a control) had such a (autoimmune) disease, type 1 diabetes.

Genotyping

DNA extraction from capillary blood samples stored at -20°C was carried out using QIAamp® Blood Kit (QIAGEN GmbH, Hilden, Germany). Instructions from the manufacturer were modified by mechanical fragmentation of the blood clot twice during the lysis procedure to maximise the DNA yield. Following storage at -20°C , the DNA samples were amplified by PCR for specific DNA sequences. The repeat (designated GT_n) in the promoter region of SLC11A1 and the microsatellite D2S1471 were PCR amplified and the products generated were sized using an Applied Biosystems 377 automated sequencer. The fluorescent oligonucleotide primers and methods used have been published previously.²⁷ The GT_n is located within a possible enhancer element containing a Z-DNA forming dinucleotide repeat.^{44,45} Four alleles have been identified in previous studies: allele 1 t(gt)₅ac(gt)₅ac(gt)₁₁g, allele 2 t(gt)₅ac(gt)₅ac(gt)₁₀g, allele 3 t(gt)₅ac(gt)₅ac(gt)₉g, and allele 4 t(gt)₅ac(gt)₉g. Only alleles 1, 2 and 3 were present in our study population. D2S1471 lies ~68 kb 3' of the SLC11A1 gene, in an intron of VIL1 [[http://www.ncbi.nlm.nih.gov/LocusLink\(VIL1\)](http://www.ncbi.nlm.nih.gov/LocusLink(VIL1))]. Thus, any haplotypes between the promoter region SLC11A1 polymorphism and D2S1471 would span the entire SLC11A1 locus.

Statistics

GraphPADInStat Version 1.15 (GPIS; University of Birmingham 921928S) statistics packages were used for assessing linkage disequilibrium between the SLC11A1 GT_n repeat and D2S1471 in the non-atopic population by Cramer's association measure (W_n) for contingency tables.⁴⁶ When $W_n = 1$, strong linkage disequilibrium is indicated; for $W_n = 0$, there is no linkage disequilibrium. The associated P -value provides a measure of confidence in the W_n statistic. Overall allele frequencies for each polymorphic locus tested were compared using the likelihood-ratio test. Haplotype frequencies were obtained using a modification of the E-M algorithm as described.⁴⁷ The likelihood-ratio tests and calculation of W_n statistics were performed within the computer programme Coda-phase written by F Dudbridge at the CIMR. Pearson's chi-squared (χ^2) test and logistic regression, using Stata 7.0 (Stata Corporation, College Station, TX, USA) were performed to assess associations between groups stratified according to BCG vaccination status and atopic phenotypes (IgE responses to food and/or airborne allergens) and allele or genotype frequencies. Odds ratios and 95% confidence intervals were estimated including adjustments for gender and age at sampling. P -values

<0.05 were considered statistically significant, however also higher *P*-values are given.

Ethics

The study was approved by the Local Research Ethics Committee and informed parental consent for the additional genetic analysis was specifically requested.

Acknowledgements

We thank A Svensson, M Grylling and A Shtauvere for technical assistance, F Lagarde for statistical advice and Pharmacia Diagnostic AB for supply of reagents for serum IgE-analyses.

References

- 1 Asher MI, Weiland SK. The International Study of Asthma and Allergies in Childhood (ISAAC). ISAAC Steering Committee. *Clin Exp Allergy* 1998; **28**: 52–66.
- 2 Cookson W. The alliance of genes and environment in asthma and allergy. *Nature* 1999; **402**: B5–11.
- 3 Romagnani S. T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol* 2000; **85**: 9–18.
- 4 Strachan DP. Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* 2000; **55**: S2–S10.
- 5 Matricardi PM, Rosmini F, Riondino S *et al*. Exposure to food-borne and orofecal microbes versus airborne viruses in relation to atopy and allergic asthma: epidemiological study. *BMJ* 2000; **320**: 412–417.
- 6 Shirakawa T, Enomoto T, Shimazu S, Hopkin JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997; **275**: 77–79.
- 7 Herz U, Gerhold K, Gruber C *et al*. BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. *J Allergy Clin Immunol* 1998; **102**: 867–874.
- 8 Wang CC, Rook GA. Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed *Mycobacterium vaccae*. *Immunology* 1998; **93**: 307–313.
- 9 Sander B, Skansén-Saphir U, Damm O, Håkansson L, Andersson J, Andersson U. Sequential production of Th1 and Th2 cytokines in response to live bacillus Calmette-Guerin. *Immunology* 1995; **86**: 512–518.
- 10 Hill AV. Genetic regulation of acquired immune responses to antigens of *Mycobacterium tuberculosis*: a study of twins in West Africa. *Infect Immun* 2001; **69**: 3989–3994.
- 11 Aaby P, Shaheen SO, Heyes CB *et al*. Early BCG vaccination and reduction in atopy in Guinea-Bissau. *Clin Exp Allergy* 2000; **30**: 644–650.
- 12 Alm JS, Lilja G, Pershagen G, Scheynius A. Early BCG vaccination and development of atopy. *Lancet* 1997; **350**: 400–403.
- 13 Strannegård IL, Larsson LO, Wennergren G, Strannegård O. Prevalence of allergy in children in relation to prior BCG vaccination and infection with atypical mycobacteria. *Allergy* 1998; **53**: 249–254.
- 14 Omenaas E, Jentoft HF, Vollmer WM, Buist AS, Gulsvik A. Absence of relationship between tuberculin reactivity and atopy in BCG vaccinated young adults. *Thorax* 2000; **55**: 454–458.
- 15 Blackwell JM, Searle S, Goswami T, Miller EN. Understanding the multiple functions of Nramp1. *Microbes Infect* 2000; **2**: 317–321.
- 16 Abel L, Sanchez FO, Oberti J, Thuc NV, Hoa LV, Lap VD. Susceptibility to leprosy is linked to the human NRAMP1 gene. *J Infect Dis* 1998; **177**: 133–145.
- 17 Bellamy R, Ruwende C, Corrah T, McAdam KP, Whittle HC, Hill AV. Variations in the NRAMP1 gene and susceptibility to tuberculosis in West Africans. *N Engl J Med* 1998; **338**: 640–644.
- 18 Cervino AC, Lakiss S, Sow O, Hill AV. Allelic association between the NRAMP1 gene and susceptibility to tuberculosis in Guinea-Conakry. *Ann Hum Genet* 2000; **64**: 507–512.
- 19 Gao PS, Fujishima S, Mao XQ *et al*. Genetic variants of NRAMP1 and active tuberculosis in Japanese populations. International Tuberculosis Genetics Team. *Clin Genet* 2000; **58**: 74–76.
- 20 Greenwood CM, Fujiwara TM, Boothroyd LJ *et al*. Linkage of tuberculosis to chromosome 2q35 loci, including NRAMP1, in a large aboriginal Canadian family. *Am J Hum Genet* 2000; **67**: 405–416.
- 21 Marquet S, Sanchez FO, Arias M *et al*. Variants of the human NRAMP1 gene and altered human immunodeficiency virus infection susceptibility. *J Infect Dis* 1999; **180**: 1521–1525.
- 22 Ryu S, Park YK, Bai GH, Kim SJ, Park SN, Kang S. 3'UTR polymorphisms in the NRAMP1 gene are associated with susceptibility to tuberculosis in Koreans. *Int J Tuberc Lung Dis* 2000; **4**: 577–580.
- 23 Shaw MA, Collins A, Peacock CS *et al*. Evidence that genetic susceptibility to *Mycobacterium tuberculosis* in a Brazilian population is under oligogenic control: linkage study of the candidate genes NRAMP1 and TNF α . *Tuber Lung Dis* 1997; **78**: 35–45.
- 24 Esposito L, Hill NJ, Pritchard LE *et al*. Genetic analysis of chromosome 2 in type 1 diabetes: analysis of putative loci IDDM7, IDDM12, and IDDM13 and candidate genes NRAMP1 and IA-2 and the interleukin-1 gene cluster. IMDIAB Group. *Diabetes* 1998; **47**: 1797–1799.
- 25 Hofmeister A, Neiberger HL, Pokorny RM, Galandiuk S. The natural resistance-associated macrophage protein gene is associated with Crohn's disease. *Surgery* 1997; **122**: 173–178.
- 26 Maliarik MJ, Chen KM, Sheffer RG *et al*. The natural resistance-associated macrophage protein gene in African Americans with sarcoidosis. *Am J Respir Cell Mol Biol* 2000; **22**: 672–675.
- 27 Sanjeevi CB, Miller EN, Dabadghao P *et al*. Polymorphism at NRAMP1 and D2S1471 loci associated with juvenile rheumatoid arthritis. *Arthritis Rheum* 2000; **43**: 1397–1404.
- 28 Shaw MA, Clayton D, Atkinson SE *et al*. Linkage of rheumatoid arthritis to the candidate gene NRAMP1 on 2q35. *J Med Genet* 1996; **33**: 672–677.
- 29 Singal DP, Li J, Zhu Y, Zhang G. NRAMP1 gene polymorphisms in patients with rheumatoid arthritis. *Tissue Antigens* 2000; **55**: 44–47.
- 30 Yang YS, Kim SJ, Kim JW, Koh EM. NRAMP1 gene polymorphisms in patients with rheumatoid arthritis in Koreans. *J Korean Med Sci* 2000; **15**: 83–87.
- 31 Blackwell JM, Searle S. Genetic regulation of macrophage activation: understanding the function of Nramp1 (=Itly/Lsh/Bcg). *Immunol Lett* 1999; **65**: 73–80.
- 32 Searle S, Bright NA, Roach TI *et al*. Localisation of Nramp1 in macrophages: modulation with activation and infection. *J Cell Sci* 1998; **111**: 2855–2866.
- 33 Goswami T, Bhattacharjee A, Babal P *et al*. Natural-resistance-associated macrophage protein 1 is an H⁺/bivalent cation antiporter. *Biochem J* 2001; **354**: 511–519.
- 34 Zwilling BS, Kuhn DE, Wikoff L, Brown D, Lafuse W. Role of iron in Nramp1-mediated inhibition of mycobacterial growth. *Infect Immun* 1999; **67**: 1386–1392.
- 35 Lang T, Prina E, Sibthorpe D, Blackwell JM. Nramp1 transfection transfers Ity/Lsh/Bcg-related pleiotropic effects on macrophage activation: influence on antigen processing and presentation. *Infect Immun* 1997; **65**: 380–386.
- 36 Soo SS, Villarreal-Ramos B, Anjam Khan CM, Hormaeche CE, Blackwell JM. Genetic control of immune response to recombinant antigens carried by an attenuated *Salmonella typhimurium* vaccine strain: Nramp1 influences T-helper subset responses and protection against leishmanial challenge. *Infect Immun* 1998; **66**: 1910–1917.
- 37 Kramnik I, Radzioch D, Skamene E. T-helper 1-like subset selection in *Mycobacterium bovis* bacillus Calmette-Guerin-infected resistant and susceptible mice. *Immunology* 1994; **81**: 618–625.
- 38 Alcais A, Sanchez FO, Thuc NV *et al*. Granulomatous reaction to intradermal injection of lepromin (Mitsuda reaction) is linked to the human NRAMP1 gene in Vietnamese leprosy sibships. *J Infect Dis* 2000; **181**: 302–308.
- 39 Compton SJ, Cairns JA, Holgate ST, Walls AF. The role of mast

- cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. *J Immunol* 1998; **161**: 1939–1946.
- 40 Dunstan SJ, Stephens HA, Blackwell JM *et al*. Genes of the class II and class III major histocompatibility complex are associated with typhoid fever in Vietnam. *J Infect Dis* 2001; **183**: 261–268.
 - 41 Medina E, North RJ. Evidence inconsistent with a role for the Bcg gene (Nramp1) in resistance of mice to infection with virulent *Mycobacterium tuberculosis*. *J Exp Med* 1996; **183**: 1045–1051.
 - 42 Medina E, North RJ. The Bcg gene (Nramp1) does not determine resistance of mice to virulent *Mycobacterium tuberculosis*. *Ann NY Acad Sci* 1996; **797**: 257–259.
 - 43 North RJ, LaCourse R, Ryan L, Gros P. Consequence of Nramp1 deletion to *Mycobacterium tuberculosis* infection in mice. *Infect Immun* 1999; **67**: 5811–5814.
 - 44 Blackwell JM, Barton CH, White JK *et al*. Genomic organization and sequence of the human NRAMP gene: identification and mapping of a promoter region polymorphism. *Mol Med* 1995; **1**: 194–205.
 - 45 Searle S, Blackwell JM. Evidence for a functional repeat polymorphism in the promoter of the human NRAMP1 gene that correlates with autoimmune versus infectious disease susceptibility. *J Med Genet* 1999; **36**: 295–299.
 - 46 Klitz W, Stephens JC, Grote M, Carrington M. Discordant patterns of linkage disequilibrium of the peptide-transporter loci within the HLA class II region. *Am J Hum Genet* 1995; **57**: 1436–1444.
 - 47 Dunstan SJ, Ho VA, Duc CM *et al*. Typhoid fever and genetic polymorphisms at the natural resistance-associated macrophage protein 1. *J Infect Dis* 2001; **183**: 1156–1160.