

## Inherited Interleukin-12 Deficiency: *IL12B* Genotype and Clinical Phenotype of 13 Patients from Six Kindreds

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Interleukin-12 (IL12) is a cytokine that is secreted by activated phagocytes and dendritic cells and that induces interferon- $\gamma$  production by natural-killer and T lymphocytes. It consists of two subunits, p35 and p40, which are encoded by *IL12A* and *IL12B*, respectively. The first reported patient with a genetic cytokine disorder was a Pakistani child, who was homozygous for a large loss-of-function deletion (g.482+82\_856–854del) in *IL12B*. This IL12-deficient child suffered from infections caused by bacille Calmette-Guérin (BCG) and *Salmonella enteritidis*. We herein report 12 additional patients from five other kindreds. In one kindred from India, the same large deletion that was described elsewhere (g.482+82\_856–854del) was identified. In four kindreds from Saudi Arabia, a recessive loss-of-function frameshift insertion (g.315\_316insA) was found. A conserved haplotype encompassing the *IL12B* gene suggested that a founder effect accounted for the recurrence of each mutation. The two founder mutational events—g.482+82\_856–854del and g.315\_316insA—were estimated to have occurred ~700 and ~1,100 years ago, respectively. Among a total of 13 patients with IL12 deficiency, 1 child had salmonellosis only and 12 suffered from clinical disease due to BCG or environmental nontuberculous mycobacteria. One patient also had clinical disease caused by virulent *Mycobacterium tuberculosis*, five patients had clinical disease caused by *Salmonella* serotypes, and one patient had clinical disease caused by *Nocardia asteroides*. The clinical outcome varies from case to case, since five patients (aged 2–11 years) died of overwhelming infection, whereas eight patients (aged 3–12 years) are still in good health and are not currently taking antibiotics. In conclusion, IL12 deficiency is not limited to a single kindred, shows significant variability of outcome, and should be considered in the genetic diagnosis of patients with mycobacteriosis and/or salmonellosis. To date, two founder *IL12B* mutations have been identified, accounting for the recurrence of a large deletion and a small insertion within populations from the Indian subcontinent and from the Arabian Peninsula, respectively.

### Introduction

Patients with Mendelian susceptibility to mycobacterial disease (MIM 209950) (McKusick 1998) are vulnerable to poorly virulent mycobacterial species—such as bacille Calmette-Guérin (BCG) vaccines (Casanova et al. 1995,

1996) and environmental nontuberculous mycobacteria (NTM) (Levin et al. 1995; Frucht and Holland 1996). They are also vulnerable to the more virulent species *Mycobacterium tuberculosis*, the agent of tuberculosis (Jouanguy et al. 1997; Altare et al. 2001). Other infectious diseases occur rarely in these patients—with the exception of salmonellosis, which affects almost half of the patients. Most sporadic and familial cases are suggestive of autosomal recessive inheritance (Levin et al. 1995; Casanova et al. 1996); however, in some families, the syndrome segregates in an autosomal dominant (Jouanguy et al. 1999; Dupuis et al. 2001) or X-linked recessive (Frucht and Holland 1996; Frucht et al. 1999) pattern. Genetic heterogeneity is further suggested by histopathological

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and clinical phenotypic heterogeneity (Emile et al. 1997). Some patients die of overwhelming mycobacterial disease with lepromatous-like lesions in early childhood, whereas other patients develop, later in life, disseminated but curable infections with tuberculoid granulomas.

Five disease-causing autosomal genes (*IFNGR1*, *IFNGR2*, *STAT1*, *IL12RB1*, and *IL12B*) have been found, and a further degree of allelic heterogeneity accounts for the existence of nine defined disorders. Although genetically different, these disorders are physiologically related, as all result in impaired interferon- $\gamma$  (IFN $\gamma$ )–mediated immunity. IFN $\gamma$  is a cytokine produced by natural-killer (NK) and T lymphocytes that binds to a heterodimeric ubiquitous receptor. Null recessive mutations in *IFNGR1* are responsible for complete IFN $\gamma$ -receptor ligand-binding-chain (IFN $\gamma$ R1) deficiency. These mutations abolish either receptor expression (Jouanguy et al. 1996; Newport et al. 1996; Pierre-Audigier et al. 1997; Altare et al. 1998b; Holland et al. 1998; Roesler et al. 1999; Cunningham et al. 2000) or the binding of surface-expressed receptors to IFN $\gamma$  (Jouanguy et al. 2000; Allende et al. 2001). Partial recessive (Jouanguy et al. 1997) and dominant (Jouanguy et al. 1999; Villella et al. 2001) IFN $\gamma$ R1 deficiencies have also been described. Different recessive mutations in *IFNGR2* are responsible for complete (Dorman and Holland 1998; Dorman et al. 2000) or partial (Döffinger et al. 2000) IFN $\gamma$  signaling-chain (IFN $\gamma$ R2) deficiency. We also recently identified a dominant mutation in *STAT1*, resulting in a partial signal transducer and activator of transcription–1 (STAT1) deficiency and in impaired cellular responses to IFN $\gamma$  (Dupuis et al. 2001).

Finally, two other genetic defects result in normal cellular responses to IFN $\gamma$  but in abnormal interleukin-12 (IL12)–dependent production of IFN $\gamma$ . Null recessive mutations have been identified in *IL12RB1*, which encodes the IL12-receptor  $\beta$ 1 subunit (IL12R $\beta$ 1) that is expressed on NK and T cells (Altare et al. 1998a; de Jong et al. 1998; Verhagen et al. 2000; Aksu et al. 2001; Altare et al. 2001; Sakai et al. 2001). As do patients with partial IFN $\gamma$ R1, IFN $\gamma$ R2, and STAT1 deficiency, patients with complete IL12R $\beta$ 1 deficiency have a milder clinical phenotype than do patients with complete IFN $\gamma$ R1 and IFN $\gamma$ R2 deficiency, probably owing to residual IFN $\gamma$ -mediated immunity (Dupuis et al. 2000). Whereas a number of patients with IL12R $\beta$ 1 deficiency have been reported, only one child has been found to carry a null homozygous large deletion in *IL12B*, which encodes the p40 subunit of IL12 (Altare et al. 1998c). IL12 is a heterodimeric cytokine (p70), comprising two subunits, p35 and p40, which is expressed by activated phagocytes and dendritic cells (Trinchieri 1998). To date, IL12 deficiency is the only human inherited cytokine disorder known. In this study, we investigated whether IL12 deficiency could be de-

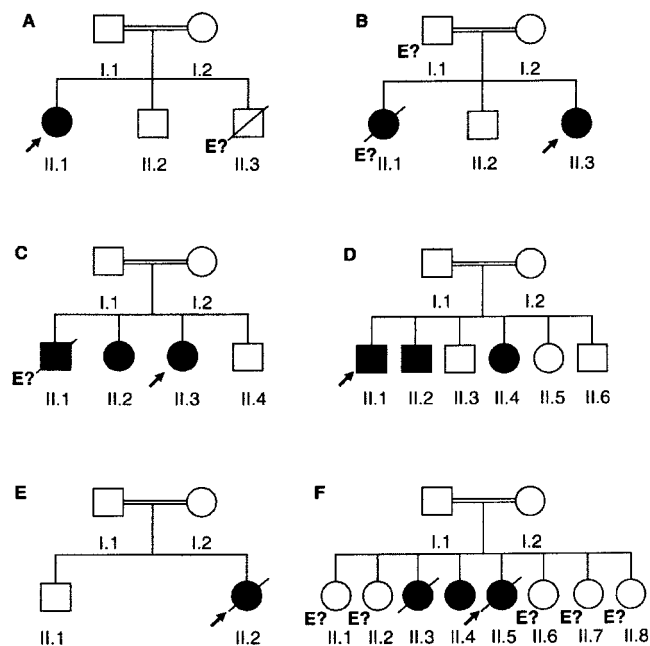
tected in other patients and, if so, whether it can be used to describe the pathogenic *IL12B* mutations involved and the associated clinical phenotypes.

### Subjects and Methods

#### Subjects and Kindreds

**Kindred A.**—The parents are consanguineous, originating from Pakistan and currently living in the United Kingdom (fig. 1 and table 1). The proband (A.II.1) suffered from BCG and *Salmonella enteritidis* infectious diseases at the ages of 3 mo and 3.5 years, respectively. She has been diagnosed with IL12 deficiency and is now 10 years old and well, with no current treatment (Altare et al. 1998c).

**Kindred B.**—The parents are first cousins, originating from the Gujarat region of India and currently living in Singapore. Their first daughter (B.II.1) was inoculated with BCG at birth and died at age 5 years, of disseminated BCG infection (Murugasu et al. 1988). A lymph-node biopsy showed tuberculoid granulomas with multiple visible acid-fast bacilli. Her brother (B.II.2), who was also vaccinated with BCG, suffered no adverse effects and is now a healthy 15 year old. The youngest sister (B.II.3) was not vaccinated with BCG. At age 2--



**Figure 1** Pedigrees of six families with IL12 deficiency caused by *IL12B* mutations. Each kindred is designated by a capital letter (A–F), each generation is designated by a roman numeral (I or II), and each individual is designated by an arabic numeral (1–8). Patients with mycobacteriosis and/or salmonellosis are represented by black symbols. The probands are indicated by arrows. “E?” indicates individuals for whom genetic analysis was not possible (e.g., B.II.1, who died of disseminated BCG infection at age 5 years).

Table 1

Clinical Phenotype of Patients with IL12 Deficiency

PATIENT	MUTATION	PLACE OF ORIGIN <sup>a</sup>	ALIVE AT FOLLOW-UP?	AGE <sup>b</sup> (years)	BACTERIA <sup>c</sup>			
					BCG	NTM	Mtb	Other
A.II.1	g.482+82_856-854del	Pakistan	Yes	11	D, R	...	...	<i>S. enteritidis</i>
B.II.1	Not done	India	No	5	D, R	...	...	...
B.II.3	g.482+82_856-854del	India	Yes	10	...	...	...	<i>S. enteritidis</i>
C.II.1	Not done	Saudi Arabia	No	2	D, R	...	...	...
C.II.2	g.315_316insA	Saudi Arabia	Yes	10	D	...	...	<i>N. asteroides</i>
C.II.3	g.315_316insA	Saudi Arabia	Yes	3	D	...	...	...
D.II.1	g.315_316insA	Saudi Arabia	Yes	11	L	...	...	...
D.II.2	g.315_316insA	Saudi Arabia	Yes	10	L	...	...	...
D.II.4	g.315_316insA	Saudi Arabia	Yes	6	L	...	L	<i>S. paratyphi C</i>
E.II.2	g.315_316insA	Saudi Arabia	No	2	D, R	...	...	...
F.II.3	g.315_316insA	Saudi Arabia	No	11	D	...	...	<i>Salmonella</i>
F.II.4	g.315_316insA	Saudi Arabia	Yes	12	D	...	...	...
F.II.5	g.315_316insA	Saudi Arabia	No	5	...	D, R	...	<i>Salmonella</i> group B

<sup>a</sup> Patient A.II.1 has been reported by Altare et al. (1998c), and patient B.II.1 has been reported by Murugasu et al. (1988).

<sup>b</sup> At death or last follow-up, at the time of preparation of the manuscript of this report.

<sup>c</sup> Mtb = *M. tuberculosis*. D = disseminated; R = recurrent; L = local. "*Salmonella*" denotes that serotype has not been determined.

4 years, she suffered four episodes of disseminated *S. enteritidis* infection, all of which responded to antibiotics. She is now 9 years old and well, with no current treatment.

**Kindred C.**—The parents are first cousins, originating from and living in Saudi Arabia. Their first child (C.II.1) was inoculated with BCG at birth and died from disseminated BCG infection at age 2 years. Their second child (C.II.2) was vaccinated with BCG shortly after birth and developed disseminated BCG infection. Lymph-node biopsy showed mature granuloma formation and multiple acid-fast bacilli. This child responded very well to antimycobacterial drugs. She presented *Nocardia asteroides* pleurisy at age 8 years and again responded well to antibiotics. The patient is now 9 years of age, in good health, and not taking medication. The proband (C.II.3) was vaccinated with live BCG at birth and developed disseminated BCG infection 6 mo later. A lymph-node biopsy showed no mature granuloma formation and multiple acid-fast bacilli. She responded well to antimycobacterial therapy and is now a healthy 2-year-old. The last child (C.II.4) was not vaccinated with live BCG and is currently a healthy 1-year-old.

**Kindred D.**—The parents are first cousins, originating from and living in Saudi Arabia. The proband (D.II.1) was vaccinated with BCG at birth and presented axillary BCG adenitis at age 14 mo. He was treated with antibiotics and by surgical excision. His brother (D.II.2) was vaccinated with live BCG at birth and presented axillary BCG adenitis at age 3 mo. Both responded well to antibiotics. The proband and his brother are now 10 and 9 years old, respectively, and are healthy, with no current treatment. Another sibling (D.II.4) was vaccinated with

live BCG at birth and presented axillary BCG adenitis 12 mo later. She was successfully treated with antibiotics and by surgical excision. At age 15 mo, she presented with *Salmonella* type C gastroenteritis. At age 2 years 6 mo, she developed occipital lymphadenitis due to *M. tuberculosis*. She was successfully treated with antibiotics but suffered a relapse at age 4 years. She is now 5 years old and well, with no antibiotic treatment. The three other siblings were vaccinated with BCG, with no adverse effect, and are currently healthy at ages 6, 3, and 1 years.

**Kindred E.**—The parents are first cousins, originating from and living in Saudi Arabia. The proband (E.II.2) was vaccinated with live BCG at birth and presented with disseminated BCG infection 8 mo later. After surgical excision, she was treated with antibiotics and INF $\gamma$ . She did not respond to treatment and later died of disseminated infection at age 2 years. Her adenitis biopsy showed tuberculoid lymphadenitis with poorly formed granuloma and numerous visible acid-fast bacilli. Her brother (E.II.1) was vaccinated with live BCG, with no adverse effect.

**Kindred F.**—The parents are first cousins, originating from and living in Saudi Arabia. The third sister (F.II.3) was vaccinated with BCG at birth and presented with disseminated BCG infection 3 mo later. She also suffered from *Salmonella* adenitis. She was treated with antibiotics for a total of 18 mo. She remained well, with no treatment, for 8 years. At age 11 years, she developed meningoencephalitis. Unfortunately, this disease proved fatal, and the causal microorganism was not isolated. Her sister (F.II.4) was vaccinated with BCG at birth and presented with disseminated BCG infection a few

months later. She was successfully treated with antibiotics. She is now 11 years old and well, with no antibiotic treatment. The proband (F.II.5) was not vaccinated with BCG at birth. She developed disseminated *M. chelonae* infection at age 3 years. She did not respond to treatment and died of disseminated infection at 5 year of age. She also presented *Salmonella* (group B) adenitis at age 3 years. Two other siblings were vaccinated with BCG, with no adverse effects, and are currently healthy at ages 16 and 13 years. The other three children were not vaccinated and are currently healthy at ages 5, 2, and 1 year. Our study was done in compliance with institutional requirements, and informed consent was obtained from each patient's family.

#### Cell Culture

Epstein-Barr virus-immortalized lymphoblastoid (EBV-B) cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 (GibcoBRL) supplemented with 10% heat-inactivated pooled fetal-calf serum (GibcoBRL). They were incubated in 24-well plates at a concentration of  $1 \times 10^6$  cells/ml with  $10^{-7}$  M phorbol-12,13-dibutyrate (PDBu) (Sigma) for 18 h. EBV-B cell lines derived from a healthy individual and from a patient previously diagnosed with IL12 deficiency (Altare et al. 1998c) were used as a positive control (C+) and as a negative control (C-), respectively. Whole-blood cells were cultured in RPMI 1640 (GibcoBRL) in 24-well plates. The cells were infected with live BCG (*M. bovis* BCG, Pasteur substrain) either at a 20:1 multiplicity of infection alone or in the presence of IFN $\gamma$  (5,000 IU/ml) or recombinant IL12p70 (20 ng/ml), for a period of 12 or 72 h.

#### Enzyme-Linked Immunosorbent Assay (ELISA)

Cell-culture supernatants were assayed for cytokines by ELISA, by use of matched antibody pairs according to the manufacturer's suggestions. ELISA reagents for human IL12p70 and IL12p40 (R&D Systems), TNF $\alpha$  (CLB), and IFN $\gamma$  (CLB) were used. Optical density was determined for each well, at 490 nm for IL12p70 and at 450 nm for IL12p40, TNF $\alpha$ , and IFN $\gamma$ , by use of an automated MR5000 ELISA reader (Dynatech). The detection limits of the assays were 0.625 pg/ml for IL12p70, 31.2 pg/ml for IL12p40, 1.4 pg/ml for TNF $\alpha$ , and 5 pg/ml for IFN $\gamma$ .

#### RNA Extraction and cDNA Synthesis

Total RNA was isolated from  $2 \times 10^7$  EBV-B cell lines, with RNazol (Biogenesis) and chloroform isoamylalcohol (Sigma). RNA was reverse transcribed by oligo (dT) with Superscript II reverse transcriptase (GibcoBRL), to produce the first-strand cDNA. In brief, 5  $\mu$ g of total RNA suspended in RNase-free water was added to 20  $\mu$ l of a reaction mixture consisting of 12.5

mM Tris-HCl, 0.75 mM MgCl<sub>2</sub>, 75 mM KCl, 5 mM DTT, and 0.5 mM each dNTP. After an initial incubation for 5 min at 42°C, 200 U of Superscript II reverse transcriptase was added. The reaction was continued for 60 min at 42°C and then was terminated by heating at 70°C for 10 min. The single-stranded cDNA was then stored at -20°C.

#### PCR Amplification

PCR amplification was performed with 1  $\mu$ l of cDNA template and 50  $\mu$ l of a reaction mixture consisting of 10 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0.2 mM each dNTP, and 1.25 U *Taq* DNA polymerase (Applied Biosystems). The primers used to amplify the coding regions of the *IL12B* and *IL12A* cDNAs (used at a concentration of 0.1  $\mu$ M) were as follows: *IL12B*, sense (5'-ggC CCA gAg CAA gAT gTg TC-3') and antisense (5'-Tgg gTC TAT TCC gTT gTg TC-3'); and *IL12A*, sense (5'-TCA CCg AgA AgC TgA TgT Ag-3') and antisense (5'-Tgg ATg TAA TAg TCC CAT CC-3'). The *IL12B* and *IL12A* cDNA coding regions were amplified by PCR under the following conditions: initial denaturation at 94°C for 5 min; followed by 40 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 90 s; and a final elongation step at 72°C for 10 min.

#### Genomic DNA Extraction and Amplification

Human genomic DNA was isolated from  $2 \times 10^7$  EBV-B cells. The cells were lysed in extraction buffer (10 mM Tris, 0.1 M EDTA, 0.5% SDS, and 10 mg proteinase K/ml) overnight at 37°C and were extracted with phenol and chloroform, and the DNA was precipitated in ethanol. PCR amplification was performed using 100 ng genomic DNA as the template and 50  $\mu$ l of a reaction mixture consisting of 10 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0.2 mM each dNTP, and 1.25 U *Taq* DNA polymerase (Applied Biosystems). The primers and conditions used for PCR amplification of the coding exons of *IL12B*, including the flanking intron sequences, are available on request.

#### Sequencing

Amplified PCR products were analyzed by gel electrophoresis in a 1% agarose gel and were purified by centrifugation through Sephadex G-50 Superfine resin (Amersham) on filter plates multiscreen MAHV-N45 (Millipore). A series of nested primers was used for sequencing (available on request). PCR products were sequenced by dideoxynucleotide termination with the BigDye terminator kit (Applied Biosystems). Sequences were analyzed on an ABI Prism 377 apparatus (Applied Biosystems).

### Northern Blotting

Total RNA (10  $\mu$ g) was separated by electrophoresis in a denaturing 1% agarose gel containing 4.5% formaldehyde at 55 V for 3 h, was transferred to a nitrocellulose membrane (Dupont-NEN), and was probed with [ $^{32}$ P]-labeled *IL12B* or *GAPDH* probes consisting of 1-kb fragments of wild-type cDNA PCR products. The probes were produced by random priming. The primers used to amplify *IL12B* were sense (5'-ggC CCA gAg CAA gAT gTg TC-3') and antisense (5'-Tgg gTC TAT TCC gTT gTg TC-3'). Transfer and hybridization were performed as described elsewhere (Jouanguy et al. 1996).

### Southern Blotting

Genomic DNA (20  $\mu$ g) was digested with *Bam*HI (GibcoBRL), was run on a 0.6% agarose gel at 55 V for 4 h, was blotted onto a nitrocellulose membrane (Dupont-NEN), and was hybridized with a [ $^{32}$ P]-labeled *IL12B* probe consisting of a 1-kb fragment of a wild-type cDNA PCR product. The primers used to amplify *IL12B* were sense (5'-ggC CCA gAg CAA gAT gTg TC-3') and antisense (5'-Tgg gTC TAT TCC gTT gTg TC-3'). Transfer and hybridization were performed as described elsewhere (Altare et al. 1998c).

### Polymorphic Marker Genotyping

Polymorphic CA repeats located in a 10,567-Mb interval around *IL12B* are available from Généthon. They were amplified, [ $^{33}$ P]-labeled, and subjected to gel electrophoresis as described elsewhere (Jouanguy et al. 1996). Three single-nucleotide polymorphisms (SNPs) in *IL12B* (-1356G/T, 364+92A/G, and 1146 C/A) were genotyped by sequencing.

### Dating of Mutations

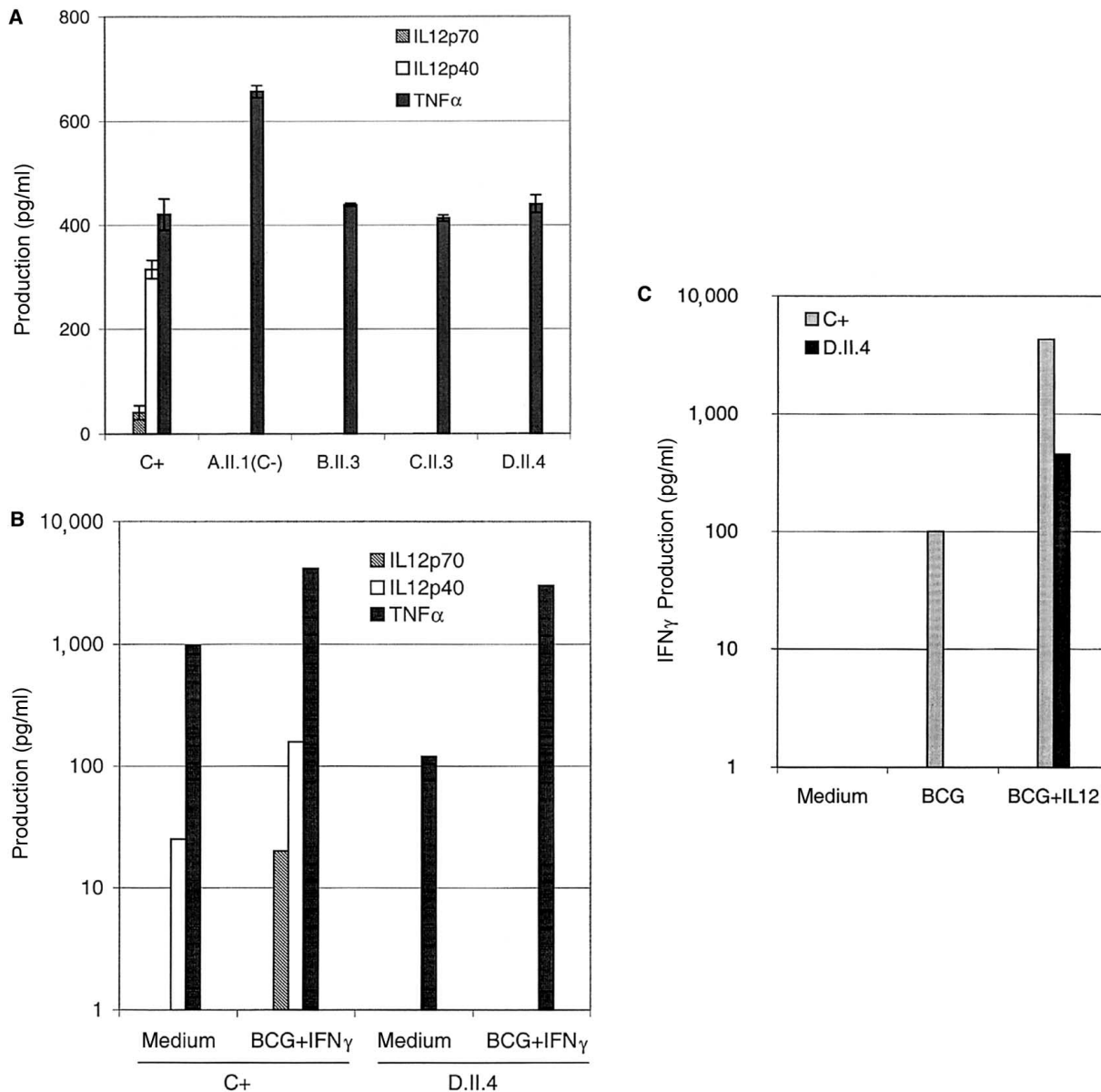
We estimated the age of the two founder mutations (g.482+82\_856-854del and g.315\_316insA) by a likelihood method that we recently developed (E.G. and L.A., unpublished data). This method assumes that the affected individuals are descended from a common ancestor in whom the mutation was introduced  $n$  generations ago. The method estimates  $n$  on the basis of the size of the haplotype shared by patients on each side of the disease locus,  $D$ , by a maximum-likelihood approach. The likelihood expression is based on two functions: (1) the probability that no crossing-over occurred during  $n$  generations between  $D$  and a marker  $x$  located at a recombination fraction  $\theta_x$  from  $D$ , a probability denoted as  $S(x) = (1 - \theta_x)^n$ , and (2) the probability that a crossing-over occurred in the  $x$ th interval between markers  $x - 1$  and  $x$  located at recombination fractions  $\theta_{x-1}$  and  $\theta_x$  (with  $\theta_{x-1} < \theta_x$ ), a probability denoted as  $S(x - 1) - S(x)$ . The method also takes into account

marker-allele frequencies, allowing for the possibility that recombination occurs not in the  $x$ th interval but in the  $(x - 1)$ th or the  $(x - 2)$ th interval, with a chromosome carrying the same allele or alleles as the ancestral haplotype at the  $(x - 1)$ th or  $(x - 2)$ th marker. Marker-allele frequencies were estimated from a sample of 32 subjects of Indian origin (g.482+82\_856-854del mutation) and 25 subjects of Arabian origin (g.315\_316insA mutation). The 95% confidence interval (95%CI) of  $n$  was obtained by a Bayesian approach, as proposed by Piccolo et al. (1996). Large simulation studies have shown that this method provides reliable estimates of  $n$  and have highlighted the importance of taking into account marker-allele frequencies in the likelihood expression (E.G. and L.A., unpublished data). Since the genetic distances available for closely linked markers are generally not very accurate, rates of recombination between markers were computed using both the overall genetic length of the haplotype and the physical distances between markers. Since the genetic and physical distances between D5S410 and D5S422, the two extreme markers of the haplotype, are 7.1 cM (Généthon Map) (Dib et al. 1996) and 10.567 Mb, respectively, as calculated by BLAST human-genome searches, the correspondence between genetic and physical distances over the whole region was estimated to be 0.672 cM for 1 Mb. Recombination fractions between the different markers and *IL12B* were then computed from genetic distances, by means of the Kosambi mapping function.

## Results

### Impaired Secretion of *IL12* in EBV-B Cells

PDBu-activated EBV-B cell lines are known to secrete detectable levels of *IL12p40* and *IL12p70* (Trinchieri 1998). We therefore tested cells derived from 70 patients with idiopathic BCG or NTM disease, for *IL12* secretion. Three activated cell lines (from patients B.II.3, C.II.3, and D.II.4) secreted no *IL12p40* and no *IL12p70* into the supernatant, as detected by ELISA (fig. 2A). Both *IL12p70* and *IL12p40* were detected in the supernatant of PDBu-activated control B cells (i.e., C+) (fig. 2A). As expected, neither *IL12p40* nor *IL12p70* was detected in the supernatant of PDBu-activated EBV-B cells from a child previously reported to have *IL12p40* deficiency (i.e., C-; A.II.1) who carried a large homozygous loss-of-function deletion (g.482+82\_856-854del) in *IL12B* (Altare et al. 1998c). Inducible TNF $\alpha$  levels confirmed that EBV-B cells from the two controls and from patients B.II.3, C.II.3, and D.II.4 were fully activated by PDBu (fig. 2A). Defective *IL12* production was observed in EBV-B cells derived from three affected siblings from kindreds C (C.II.2) and D (D.II.1 and D.II.2) (data not shown). EBV-B cells were not available from



**Figure 2** Impaired production of IL12. *A*, Production of IL12p70, IL12p40, and TNF $\alpha$ , by PDBu-stimulated EBV-B cells from a healthy positive control (C+) and a negative control (C-; A.II.1) (both of whom had previously been reported by Altare et al. [1998c]) and from patients (B.II.3, C.II.3, and D.II.4). Supernatants were harvested after 18 h of activation, for cytokine quantification by ELISA. The results are the mean  $\pm$  SDs of two experiments. *B*, Production of IL12p70, IL12p40, and TNF $\alpha$ , by whole-blood cells from a healthy positive control (C+) and from patient D.II.4, either unstimulated (Medium) or stimulated with BCG plus IFN $\gamma$ . Supernatants were harvested after 12 h of activation. *C*, Production of IFN $\gamma$  by whole-blood cells from healthy positive control (C+) and from patient D.II.4, either unstimulated (Medium) or stimulated with BCG alone or with BCG plus recombinant IL12p70. The supernatants were harvested after 72 h of activation, for cytokine quantification by ELISA.

affected siblings B.II.1 and C.II.1. These results suggest that at least six patients, from kindreds B–D, had IL12 deficiency.

*Impaired Secretion of IL12 in Blood Cells*

To validate the results obtained with EBV-B cells, we measured the production of IL12p40 and IL12p70 by

whole-blood cells from patient D.II.4; the cells were either unstimulated or stimulated with live BCG plus IFN $\gamma$  (fig. 2*B*). Neither IL12p70 nor IL12p40 was detected by ELISA. In contrast, both IL12p70 and IL12p40 were detected in the control-cell supernatant (i.e., C+) (fig. 2*B*). Inducible TNF $\alpha$  levels confirmed that blood cells from the control and patient D.II.4 were fully activated

(fig. 2B). Similar results were obtained if cells were stimulated with BCG alone (not shown). In a previous experimental study, we had shown that neither IL12p40 nor IL12p70 was detectable in the supernatant of similarly activated blood cells derived from patient A.II.1 (i.e., C<sup>-</sup>) (Altare et al. 1998c). We therefore measured IFN $\gamma$  production by blood cells from a control (i.e., C<sup>+</sup>) and from patient D.II.4, the cells being either unstimulated or stimulated by live BCG or BCG plus IL12p70 (fig. 2C). The addition of recombinant exogenous IL12p70 led to an increase in IFN $\gamma$  production in the patient's blood cells, to almost normal levels, in response to BCG challenge (fig. 2C). This experiment shows that the lack of detectable IL12 accounts for the poor production of IFN $\gamma$ .

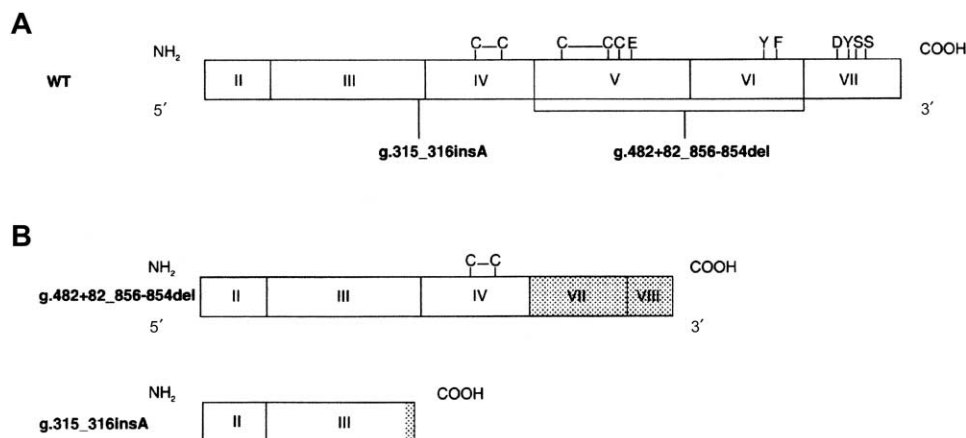
#### A Large Deletion in IL12B

A cDNA was synthesized from mRNA derived from the EBV-B cells of patient B.II.3. This cDNA was used to amplify and sequence the *IL12A* and *IL12B* coding regions. A large frameshift deletion of 373 nt was detected between positions 482 and 856 in *IL12B* (not shown). The sequence of the coding region of the *IL12A* gene displayed no mutation. Genomic DNA was used as the template for PCR amplification of the eight *IL12B* exons and flanking intron regions. A homozygous 4.6-kb frameshift deletion mutation encompassing coding exons V and VI was identified and designated "482+82\_856-854del" (fig. 3) (Dunnen and Antonarakis 2000). This deletion is identical to that found in the Pakistani patient (A.II.1) who elsewhere had been reported to have IL12p40 deficiency (Altare et al. 1998c) and was not found in 174 unrelated healthy controls from the Indian subcontinent (59 of whom were from the Gujarat region). This indi-

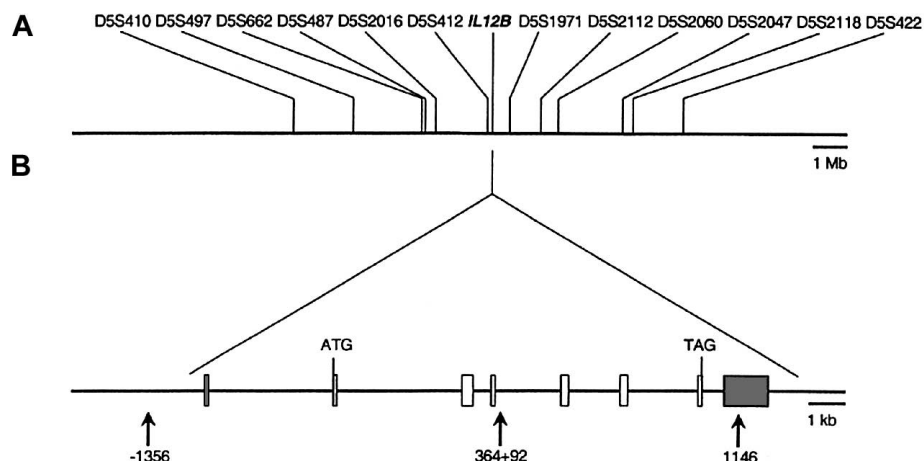
cates that the frequency of this deletion can be estimated (with a  $P > .95$ ) to be  $< 0.009$ . By northern blot analysis, *IL12B* transcripts were not detected in B.II.3 and A.II.1 (i.e., C<sup>-</sup>) EBV-B cells, in contrast to those of the positive control (not shown). This mutation results in the loss of 167 of the original 328 amino acids and in the addition of 45 new amino acids to the COOH-terminal region of the mature polypeptide. In a previous experimental study, we had demonstrated that the g.482+82\_856-854del mutation is a loss-of-function mutation, by transfecting a B cell line from the patient with the mutant and a wild-type *IL12B* allele (Altare et al. 1998c). As in kindred A (Altare et al. 1998c), we showed by Southern blot analysis that patient B.II.3 was homozygous—and that her healthy brother and mother were heterozygous—for the deletion, strongly suggesting that the deletion is recessive in kindred B (not shown). DNA from the father and from the first, deceased child (B.II.1) was not available for analysis.

#### A Small Insertion in IL12B

Sequencing of the coding region of the *IL12B* cDNA from patients C.II.3 and D.II.4 revealed the insertion of an adenosine between positions 315 and 316 (not shown). No mutation was observed in the sequence of the coding region of *IL12A*. Genomic amplification and sequencing of the *IL12B* exon III confirmed that the mutation (designated "g.315\_316insA") was homozygous (fig. 3). This mutation was not found in 115 Arabian healthy unrelated controls analyzed (73 of whom were from the Arabian Peninsula). This indicates that the frequency of this insertion can be estimated (with a  $P > 0.95$ ) to be  $< 0.012$ . The frameshift results in a premature stop codon at nucleotide positions 342–344. By



**Figure 3** *IL12B* mutant alleles. *A*, Schematic representation of structural organization of coding region of wild-type (WT) *IL12B* gene. Exons I and VIII are not translated. Intrachain disulfite bonds and amino acids essential for dimerization with IL12p35 are indicated by the single-letter amino acid code. The positions of the two mutations found in IL12-deficient patients are also indicated. *B*, Schematic representation of coding region of two mutant *IL12B* alleles. Gray regions correspond to stretches of new amino acids resulting from the frameshift mutation.



**Figure 4** High-resolution map of *IL12B* locus. *A*, Physical map of *IL12B* region on chromosome 5q33. The physical distances (in Mb) between *IL12B* and microsatellite markers were obtained from BLAST. *B*, Genomic organization of human *IL12B* gene and location of intragenic SNPs. White boxes indicate coding exons; gray boxes indicate untranslated exons. Translation initiation and termination codons are indicated. Arrows indicate positions of intragenic SNPs (–1356, 364+92, and 1146).

northern blot analysis, *IL12B* transcripts were not detected in PDBu-stimulated EBV-B cells from patients C.II.3 and D.II.4. *IL12B* transcripts were detected in PDBu-stimulated EBV-B cells from a control (i.e., C+) (not shown). This mutation results in the loss of 223 of the original 328 amino acids and in the addition of 9 new C-terminal amino acids. The encoded protein lacks all the amino acids shown by crystallography to be essential for p40 dimerization with p35 (fig. 3) (Yoon et al. 2000). Healthy relatives were found to be either heterozygous for the g.315\_316insA mutant allele or homozygous for a wild-type allele. These data strongly suggest that the g.315\_316insA mutation accounts for the lack of detectable p40 and p70 and is a loss-of-function recessive mutation.

#### *IL12* Deficiency in Other Kindreds

We next sequenced *IL12B* exons in 16 patients selected from 4 kindreds from the Indian subcontinent and from 10 kindreds from the Arabian Peninsula, for whom EBV-B cells were not available. We identified four patients (E.II.2, F.II.3, F.II.4, and F.II.5) from two new kindreds (E and F) from Saudi Arabia that carried the homozygous mutation g.315\_316insA (fig. 1). No mutations were found in the other patients. Parents and healthy siblings from kindreds C–F were heterozygous for g.315\_316insA (fig. 1). DNA from the first deceased sibling (B.II.1) of kindred B and from the healthy siblings (F.II.1, F.II.2, F.II.6, F.II.7, and F.II.8) of kindred F were not available for analysis. No other mutations were found in the coding region of *IL12B*. In total, nine patients from four kindreds (C–F) from Saudi Arabia were found to be homozygous for an identical loss-

of-function *IL12B* frameshift insertion (g.315\_316insA) (fig. 1).

#### *A Founder Effect Accounts for the Recurrence of These Mutations*

Polymorphic markers encompassing the *IL12B* gene on 5q31.1–5q33, including flanking dinucleotide repeats (D5S410, D5497, D5S662, D5S487, D5S2016, D5S412, D5S1971, D5S2112, D5S2060, D5S2047, D5S2118, and D5S422) and intragenic SNPs (–1356 G/T, 364+92 A/G, and 1146 C/A) were genotyped in all patients (fig. 4). A 3.6-Mb homozygous haplotype defined by the three intragenic SNPs (not shown) and by seven microsatellites (D5S662–D52060) is shared by all affected siblings from kindreds A and B (who are homozygous for the g.482+82\_856–854del mutation) (fig. 5). Patients from kindreds C–F (homozygous for the g.315\_316insA mutation) share a 0.516-Mb common homozygous haplotype defined by the three intragenic SNPs (not shown) and two microsatellites (D5S412 and D5S1971) (fig. 5). These data strongly suggest that each mutation results from a founder event. We developed a new mutation-dating method to date both the closest ancestor common to all patients from kindreds A and B and the closest ancestor to all patients from kindreds C–F (E.G. and L.A., unpublished data). Indeed, methods described elsewhere (Guo and Xiong 1997; McPeck and Strahs 1999; Slatkin and Rannala 2000) do not apply to very small numbers of kindreds. For the g.482+82\_856–854del mutation, the estimated number of generations since the appearance of the common ancestor is 29 (95% CI 9–115). If it is assumed that a generation is



Markers	D5S410	D5S497	D5S662	D5S487	D5S2016	D5S412	<i>IL12B</i>	D5S1971	D5S2112	D5S2060	D5S2047	D5S2118	D5S422
Mb	153.3	154.9	156.8	157.1	157.3	158.8	158.9	159.2	160	160.4	162.3	162.5	163.8
$\theta$	.0372	.0264	.0136	.0113	.0108	.0008	0	.0026	.0080	.0106	.0230	.0245	.0333
A.II.1	4/4	1/1	3/3	3/3	1/1	6/6	g.482+82_856-854del	3/3	7/7	3/3	4/4	1/2	1/2
B.II.3	2/2	1/6	3/3	3/3	1/1	6/6	g.482+82_856-854del	3/3	7/7	3/3	5/5	2/2	3/3
C.II.2	1/5	1/5	4/3	3/3	2/2	5/5	g.315_316insA	1/1	6/1	3/3	4/4	3/4	3/3
C.II.3	1/5	1/5	4/3	3/3	2/2	5/5	g.315_316insA	1/1	6/1	3/3	4/4	3/4	3/3
D.II.1	6/6	5/5	2/2	5/5	2/2	5/5	g.315_316insA	1/1	4/4	5/5	4/4	4/4	3/3
D.II.2	6/6	5/5	2/2	5/5	2/2	5/5	g.315_316insA	1/1	4/4	5/5	4/4	4/4	3/3
D.II.4	6/6	5/5	2/2	5/5	2/2	5/5	g.315_316insA	1/1	4/4	5/5	4/4	4/4	3/3
E.II.2	4/4	1/1	6/6	3/3	1/1	5/5	g.315_316insA	1/1	4/4	3/3	4/4	4/4	3/3
F.II.3	6/6	1/1	6/6	3/3	1/1	5/5	g.315_316insA	1/1	4/4	3/3	4/4	4/4	4/4
F.II.4	6/6	1/1	6/6	3/3	1/1	5/5	g.315_316insA	1/1	4/4	3/3	4/4	4/4	4/4
F.II.5	6/6	1/1	6/6	3/3	1/1	5/5	g.315_316insA	1/1	4/4	3/3	4/4	4/4	4/4

**Figure 5** Haplotypes encompassing *IL12B* in patients with IL12 deficiency. Markers encompassing *IL12B*, with physical distance (Mb) and recombination rate ( $\theta$ ), are shown. Physical distance was obtained from BLAST; the recombination rate was estimated by use of the correspondence of .672 cM for 1 Mb, over the whole region, and use of the Kosambi mapping function (see the “Dating of Mutations” subsection). The ancestral haplotypes shared by kindreds A and B, from the Indian subcontinent, is indicated by dark-gray shading; the ancestral haplotype shared by kindreds C–F, from Saudi Arabia, is indicated by intermediate-gray shading; and the ancestral haplotype shared by a subset of kindreds is indicated by light-gray shading.

~24 years, then the g.482+82\_856–854del mutation in *IL12B* is ~700 years old (95%CI 216–2,760). For the g.315\_316insA mutation, the estimated number of generations is 47 (95%CI 22–110), indicating that this mutation in *IL12B* is ~1,100 years old (95%CI 528–2,640).

## Discussion

We report herein 12 patients from five new kindreds with complete IL12 deficiency associated with *IL12B* mutations. Our study illustrates that, although rare, this human cytokine gene disorder is not limited to the single patient who has been described elsewhere (Altare et al. 1998c). Twelve of the 13 patients with IL12 deficiency were found to suffer from mycobacteriosis, and 5 were found to suffer from salmonellosis, which was the sole clinical manifestation in 1 child. Despite an overall better clinical outcome than that had by patients with complete IFN $\gamma$ R deficiency, interfamilial heterogeneity was observed. In one kindred from India, we found a large deletion that was previously reported in a kindred from Pakistan (Altare et al. 1998c), whereas, in four kindreds from Saudi Arabia, we identified a new recessive loss-of-function *IL12B* small insertion. Each rare mutation resulted from a founder effect in either the Indian subcontinent (large deletion) or the Arabian Peninsula (small insertion), and, by a new method, we were able to date those original mutation events to ~700 and ~1,100 years ago, respectively.

This study confirms that IL12 deficiency is principally

associated with infectious diseases caused by *Mycobacteria* (table 1). All but one of the patients reported here have had at least one episode of mycobacterial infection. All children inoculated with live BCG vaccine developed clinical infection, as did the previously reported IL12-deficient patient (Altare et al. 1998c). One patient who was not inoculated with BCG developed NTM infection (a fast-growing species, *M. chelonae*). One child with BCG infection subsequently developed *M. tuberculosis* adenitis. Most patients with IL12R $\beta$ 1 deficiency are susceptible to BCG (Altare et al. 1998a; de Jong et al. 1998; Verhagen et al. 2000), and two children who were apparently resistant to BCG developed *M. chelonae* (Aksu et al. 2001) and *M. tuberculosis* (Altare et al. 2001) infections. Another NTM, *M. avium*, which was detected in IL12R $\beta$ 1-deficient patients not previously inoculated with BCG (de Jong et al. 1998; Verhagen et al. 2000; Sakai et al. 2001), has not, to date, been diagnosed in the series of IL12-deficient patients whom we studied. Experimental studies have shown that IL12 is essential for protective immunity against *Mycobacteria* in mice. Animals with disrupted *IL12B* genes are highly susceptible to BCG (Cooper et al. 1997; Wakeham et al. 1998), *M. avium* (Doherty and Sher 1998; Silva et al. 2001), and *M. tuberculosis* (Magram et al. 1996). Our results extend these observations, highlighting the crucial role that IL12 plays in antimycobacterial immunity in mice and humans. Clinically, IL12 deficiency should be considered in the genetic diagnosis of patients with BCG or NTM severe diseases. Further studies are required to investigate the role that rare and

frequent *IL12B* mutations play in susceptibility to tuberculosis in the general population (Abel and Casanova 2000).

Together with our previous report (Altare et al. 1998c), this study suggests that the course of mycobacterial infection in patients with complete IL12 deficiency is less severe than that in patients with complete IFN $\gamma$ R deficiency (Jouanguy et al. 1996; Newport et al. 1996; Pierre-Audigier et al. 1997; Dorman and Holland 1998; Roesler et al. 1999; Cunningham et al. 2000; Jouanguy et al. 2000; Allende et al. 2001). Only 5 of the 13 IL12-deficient patients (aged 2–11 years) died of infection; the 8 survivors (aged 3–12 years) responded well to antibiotics and are now well and not currently taking medication (i.e., for 2–9 years have not undergone treatment). Granuloma formation is preserved but often multibacillary in patients with IL12 deficiency, whereas patients with complete IFN $\gamma$ R deficiency are not able to form mature granulomas. However, there is considerable interfamilial heterogeneity in terms of outcome. Nine patients have presented disseminated mycobacterial infection, which has proved fatal in four cases, whereas, in three children from the same kindred, BCG infection was clinically limited to the draining lymph nodes. Accordingly, the clinical outcome of IL12R $\beta$ 1 deficiency also differs greatly between patients, with two deaths reported (at age 5–8 years) and 10 survivors (aged 7–31 years) well and not currently taking medication (Altare et al. 1998a; de Jong et al. 1998; Verhagen et al. 2000; Aksu et al. 2001; Altare et al. 2001; Sakai et al. 2001).

Five patients have presented clinical *Salmonella* infection, as did the IL12-deficient patients described by Altare et al. (1998c) and 6 of 12 IL12R $\beta$ 1-deficient patients reported elsewhere (Altare et al. 1998a; de Jong et al. 1998; Verhagen et al. 2000). This is a well-known feature of the syndrome of Mendelian susceptibility to mycobacterial disease (Levin et al. 1995; Casanova et al. 1996). Other severe infections caused by *Listeria monocytogenes* (Roesler et al. 1999), *Histoplasma capsulatum* (Jouanguy et al. 1999), and viruses (Dorman et al. 1999; Cunningham et al. 2000; Uzel et al. 2000), which elsewhere have been reported in patients with IFN $\gamma$ R deficiency, were not diagnosed in patients with IL12 or IL12R deficiency; however, we report, for the first time, a case of clinical *N. asteroides* infection in a patient with an impaired IL12-IFN $\gamma$  axis. It is difficult to determine whether there is a causal relationship between impaired IL12-mediated immunity and nocardiosis. Such a relationship is probable, however, given the considerable biochemical and phylogenetic similarity between *Mycobacterium* and *Nocardia*, which form, together with *Corynebacterium*, a monophyletic taxon

of mycolic acid-containing acid-fast gram-positive bacteria (Embley and Stackebrandt 1994).

A novel heterodimeric cytokine, interleukin-23 (IL23), has recently been described (Oppmann et al. 2000). This cytokine consists of the p40 subunit of IL12 and a novel p19 subunit, encoded by a gene mapping to 12q13. IL23 is produced by activated mature dendritic cells and induces IFN $\gamma$  production by memory T lymphocytes in humans and in mice. The patients with IL12p40 deficiency described herein therefore probably suffer not only from IL12 deficiency but also from IL23 deficiency. Further studies with hIL23-specific antibodies are necessary to investigate whether this is indeed the case. If IL23 deficiency is established in these patients, it will be important to discriminate between the specific contributions of IL12 and IL23 deficiencies to the development of mycobacterial disease in patients with *IL12B* mutations. IL12p35-deficient mice have a phenotype milder than do IL12p40-deficient mice, in terms of vulnerability to *Leishmania major* (Mattner et al. 1996) and to *Cryptococcus neoformans* (Decken et al. 1998), suggesting that IL23 may perhaps contribute to protection against mycobacteria. The identification of IL12p35-deficient patients with a milder phenotype than that of IL12p40-deficient patients would support this hypothesis.

Only two *IL12B* mutant alleles accounted for IL12 deficiency in the six kindreds identified. A large deletion was found in patients from the Indian subcontinent, and a small insertion was found in patients from the Arabian Peninsula. The Pakistani child and the Indian child, both originating from the northern region of the Indian subcontinent, presented a large deletion (g.482+82\_856–854del) encompassing exons V and VI (Altare et al. 1998c). A 3.6-Mb homozygous haplotype encompassing *IL12B* is shared by the two patients, implying that a founder effect is involved. The g.315\_316insA mutation, which was found in four unrelated kindreds from Saudi Arabia, has not previously been described and the 0.516-Mb homozygous haplotype shared by the four kindreds is also strongly suggestive of a founder effect. These are the first two examples of founder effects in genetic defects of the IL12-IFN $\gamma$  axis, which predispose the individual to mycobacterial disease. The closest common ancestor carrying the founder allele in each region was dated by a new method (E.G. and L.A., unpublished data), which was developed for use with very small numbers of kindreds. The common ancestor of patients carrying the Indian deletion was estimated to have lived ~700 years ago, whereas the common ancestor of patients carrying the Saudi Arabian insertion was estimated to have lived ~1,100 years ago. The two *IL12B* mutant alleles are probably rare (frequency < .01) in the respective populations, because they were not found in all healthy in-

dividuals genotyped. Novel homozygous cases are, however, likely to be identified in the future, particularly in more-specific regions (e.g., northern India–Pakistan and Saudi Arabia). A direct clinical implication of this study is that well-defined *IL12B* mutations should be sought as a matter of priority in patients with mycobacteriosis and salmonellosis who originate from either the Indian subcontinent or the Arabian Peninsula.

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## Electronic-Database Information

The accession number and URLs for data in this article are as follows:

- BLAST, <http://www.ncbi.nlm.nih.gov/BLAST/> (for localization of microsatellites markers from the chromosome 5 and *IL12B* sequences)  
 Génethon Map, [ftp://ftp.genethon.fr/pub/Gmap/Nature-1995/data/data\\_chrom5](ftp://ftp.genethon.fr/pub/Gmap/Nature-1995/data/data_chrom5) (for microsatellites markers from the chromosome 5 region)  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for familial atypical mycobacteriosis [MIM 209950])

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