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Research Article

Interferon-gamma receptor ligand-binding chain (IFN-gammaR1) or signaling chain (IFN-gammaR2) deficiency, like interleukin 12 receptor beta1 chain (IL-12Rbeta1) deficiency, predispose to severe infections due to poorly virulent mycobacteria and salmonella. A child with bacille Calmette-Guérin and Salmonella enteritidis infection was investigated. Mutations in the genes for IFN-gammaR1, IFN-gammaR2, IL-12Rbeta1, and other molecules implicated in IL-12- or IFN-gamma-mediated immunity were sought. A large homozygous deletion within the IL-12 p40 subunit gene was found, precluding expression of functional IL-12 p70 cytokine by activated dendritic cells and phagocytes. As a result, IFN-gamma production by lymphocytes was markedly impaired. This is the first discovered human disease resulting from a cytokine gene defect. It suggests that IL-12 is essential to and appears specific for protective immunity to intracellular bacteria such as mycobacteria and salmonella.

Inherited Interleukin 12 Deficiency in a Child with Bacille Calmette-Guérin and *Salmonella enteritidis* Disseminated Infection

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Abstract

Interferon-γ receptor ligand-binding chain (IFN-γR1) or signaling chain (IFN-γR2) deficiency, like interleukin 12 receptor β1 chain (IL-12Rβ1) deficiency, predispose to severe infections due to poorly virulent mycobacteria and salmonella. A child with bacille Calmette-Guérin and *Salmonella enteritidis* infection was investigated. Mutations in the genes for IFN-γR1, IFN-γR2, IL-12Rβ1, and other molecules implicated in IL-12– or IFN-γ–mediated immunity were sought. A large homozygous deletion within the IL-12 p40 subunit gene was found, precluding expression of functional IL-12 p70 cytokine by activated dendritic cells and phagocytes. As a result, IFN-γ production by lymphocytes was markedly impaired. This is the first discovered human disease resulting from a cytokine gene defect. It suggests that IL-12 is essential to and appears specific for protective immunity to intracellular bacteria such as mycobacteria and salmonella. (*J. Clin. Invest.* 1998. 102:2035–2040.) Key words: immunodeficiency • mycobacterium • granuloma • dendritic cell • macrophage

Introduction

Bacille Calmette-Guérin (BCG) vaccines (attenuated substrains of *Mycobacterium bovis*) and environmental nontuberculous mycobacteria are poorly pathogenic mycobacterial species in humans. These intracellular bacteria are leading causes of disseminated disease in patients with severe immunodeficiencies. However, they may also cause disseminated infection in otherwise healthy individuals with no well-defined immunodeficiency state (1–4). Disseminated infections due to non-typhi salmonella, another group of poorly virulent intracellular bacteria, occur in approximately half of the cases, but other viral, prokaryotic, and eukaryotic microorganisms do not appear to cause clinical disease in these children.

Characterization of complete IFN-γ receptor ligand-binding chain (IFN-γR1)[1] deficiency provided the first genetic etiology for this syndrome (5–9). Mutations in the IFN-γ receptor signaling chain (IFN-γR2) were also identified in another kindred (10). These two conditions highlighted the essential role of IFN-γ, a pleiotropic cytokine secreted by T and natural killer (NK) lymphocytes, in the control of mycobacteria. Mature granulomas, with epithelioid and multinucleated cells surrounded by lymphocytes, were not seen in these children. This suggests that both phagocytes and lymphocytes, constitutively deprived of IFN-γ stimulation, are implicated in the pathogenesis of mycobacterial infection. The prognosis of affected children is poor, with early onset and often fatal mycobacterial infection.

Several patients with this syndrome have a milder clinical and histopathological phenotype, and this was found to reflect the underlying genetic defect. Two siblings with partial, as opposed to complete, IFN-γR1 deficiency were first identified (11). Recently, IL-12 receptor β1 chain (IL-12Rβ1) deficiency was identified in other kindreds (12, 13). These patients were found to have impaired, yet not abrogated, IFN-γ secretion, and mature BCG granulomas were seen. Some patients were asymptomatic until adulthood and mycobacterial infections were often curable. Herein, we report the identification of IL-12 deficiency in a child with curable BCG and *Salmonella enteritidis* infection.

Methods

Case report. A girl born to consanguineous Pakistani parents received BCG immunization at birth. She presented 3 mo later with local ulceration of her immunization site on her left deltoid region, regional lymphadenopathy, and a discharging sinus from which *M. bovis* BCG was isolated. The granulation tissue and an underlying axillary lymph node were excised. Histological examination revealed

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1. *Abbreviations used in this paper:* ESR, erythrocyte sedimentation rate; IFN-γR1, IFN-γ receptor ligand-binding chain; IFN-γR2, IFN-γ receptor signaling chain; IL-12Rβ1, IL-12 receptor β1 chain; NK, natural killer.

widespread macrophages and polymorphonuclear neutrophils in the dermis and in the lymph node, with rare multinucleated phagocytes, but without any well-circumscribed tuberculoid granulomatous lesion consisting of epithelioid and Langhans' cells surrounded by lymphocytes. All phagocytic cells were loaded with acid-fast bacilli (Fig. 1 *A*). These lesions can be depicted as type II BCG granulomas in the context of idiopathic infection (4). Despite poor granuloma formation in the tissue sample, no clinical signs of BCG dissemination were noted. She was treated with rifampicin, isoniazid, and PAS for 15 mo and initially did well. At 2 yr of age, the axillary lymph nodes were found to be persistently enlarged and tender and were surgically excised. Histology at this stage showed moderately well-circumscribed and -differentiated tuberculoid granulomas with epithelioid cells and Langhans' cells, surrounded by lymphocytes (Fig. 1 *B*). The lesions, which were paucibacillary, can be depicted as type I BCG granulomas in the context of idiopathic infection (4). *M. bovis* BCG, which was fully sensitive to the antimycobacterial agents used previously, was again isolated from this second lymph node biopsy. Antimycobacterials were continued for a further 6 mo. 6 mo later (at 3 yr of age) the child developed further lymph node enlargement in the contralateral axilla (right side) and left upper cervical region as well as hepatomegaly, 5 cm below costal margin. The erythrocyte sedimentation rate (ESR) was 88 mm/h and the hemoglobin was reduced to 9 g/dl. Fully drug sensitive *M. bovis* BCG was again isolated from a percutaneous liver biopsy, and antimycobacterials were continued. At 3.5 yr of age, the child also developed severe gastroenteritis, with bloody diarrhea and septicemia due to *S. enteritidis* (blood and stool cultures positive). The child remained ill despite several courses of antibiotic therapy. She was then treated with a combination of antituberculous therapy, cotrimoxazole-trimethoprim, and subcutaneous IFN-γ at a dose of 50 μg/m^2 three times a week. A marked symptomatic improvement was observed after IFN-γ therapy was commenced. At 4 yr of age, antimycobacterial drugs were discontinued. At the end of a further year, IFN-γ was stopped but she was continued on cotrimoxazole-trimethoprim. Within 3 mo the child developed large submandibular lymph nodes (4–5 cm), fever, and elevated ESR (55 mm/h). A lymph node biopsy showed reactive hyperplasia, and no mycobacteria could be cultured, but *S. enteritidis* of the same phage type (type 8) and antibiotic sensitivity pattern as the strain isolated previously (at 3.5 yr of age) from her blood culture was isolated from this tissue. IFN-γ therapy was recommenced, in addition to cotrimoxazole-trimethoprim, and when recently reviewed at 8 yr of age, she remains well. The course of common childhood infections were unremarkable and no other opportunistic infections were documented. Elevated titers of IgG specific for EBV, Varicella-Zoster virus, and Herpes Simplex 1 virus were detected in the serum. No specific antibodies against *Toxoplasma gondii* and cytomegalovirus were found. No symptoms of atopy were detected. One brother has been vaccinated with BCG with no adverse effect, and another, born in Pakistan, died at the age of 1 yr with fever of unknown cause (no material was available for genetic analysis). Interestingly, the father suffered in childhood of severe and recurrent nontyphi salmonella (*Salmonella bareilly*) infection requiring several prolonged courses of antibiotic therapy over 4 yr, and the paternal grandmother had pulmonary tuberculosis, which was successfully treated with antituberculous therapy.

Molecular genetics. Extraction of total RNA from PBMC or EBV-transformed B cells, cDNA synthesis, PCR, and sequencing were performed as described (12). Primers for amplification of IL-12 p40 cDNA coding region (14) were sense 5′-GGC CCA GAG CAA GAT GTG TC-3′ and antisense 5′-TGG GTC TAT TCC GTT GTG TC-3′. A series of nested primers was used for sequencing (available upon request). Northern blot analysis was performed as described (6) using as a probe a [^{32}P-α]dCTP-labeled cDNA-PCR product of IL-12 p40 encompassing the entire coding region. Extraction of genomic DNA, PCR, and sequencing were also performed as described previously (12); a series of nested primers based on the published sequence of the cDNA was synthesized to determine the genomic structure of the *IL12P40* gene and for amplification of the deletion and recombination breakpoints (available upon request). For Southern blot, genomic DNA was digested with restriction enzyme BamHI, run on an agarose gel, blotted onto a nylon membrane, and hybridized to a ^{32}P-labeled *IL12P40* probe consisting of a wild-type cDNA PCR product. For transfection studies, 5×10^6 EBV-transformed B cells were electroporated with 10 μg pREP4 expression vector containing either *IL12P40* del4.4 cDNA (p40del4.4) or *IL12P40* wild-type cDNA (p40wt), in 800 μl of RPMI 1640 supplemented with 20% FCS. One pulse was delivered (250 V, 1,500 μF) with a Cellject electropulser and the cells were placed in 5 ml selective complete medium (RPMI 1640, 10% FCS, hygromycin 250 μg/ml). After 1 mo, the supernatants of transfectant lines were tested by ELISA for the presence of IL-12. The supernatant was concentrated 10-fold by filtration through Centricon 50 before quantification of IL-12 p70. Alternatively, the supernatant and/or PHA was used to induce IFN-γ by PBMC (see below).

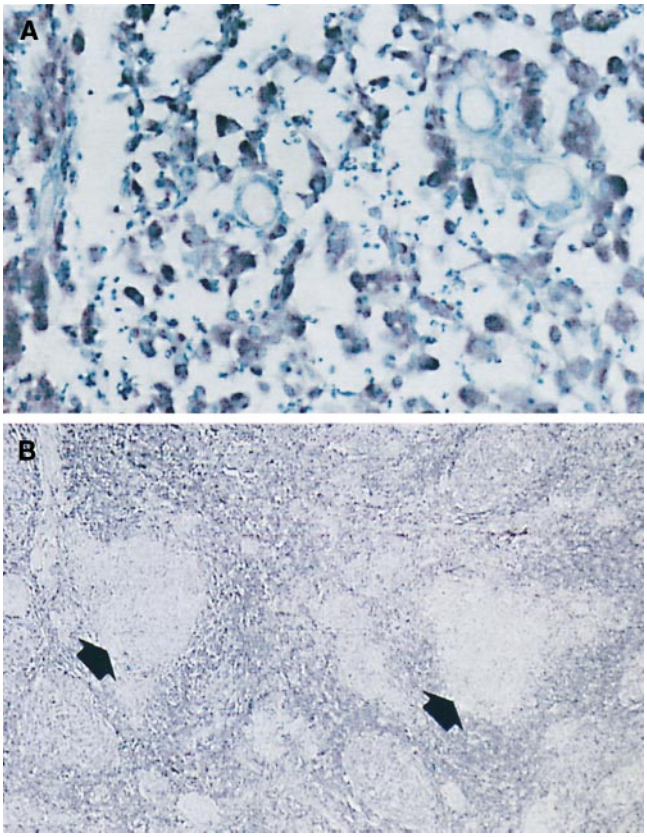

Figure 1. BCG granulomas. (*A*) Axillary lymph node poorly defined and multibacillary granulomas observed 3 mo after inoculation of BCG in the deltoid region (Ziehl-Nilsen, ×400). (*B*) Axillary lymph node well-circumscribed and -differentiated granulomas (*arrows*) observed 15 mo after antibiotic therapy was commenced (hematoxylin and eosin, ×100).

Cellular immunology. To quantify IL-12 production, PBMC were purified by Ficoll-Hypaque density gradient separation and cultured in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated pooled human AB$^+$ serum (National Blood Transfusion Service, Birmingham, UK) in 24-well plates at a concentration of 2×10^6/ml. The cells were infected with live BCG substrain Evans for 12 or 24 h at a 10:1 moi. Supernatants were harvested at 12 h for IL-12 p70 and p40 quantification by ELISA (R&D Systems, Inc., Minneapolis, MN) and at 24 h for TNF-α (R&D Systems, Inc.). To quantify IFN-γ production, PBMC cultured in the same conditions were stimulated with live BCG (moi 10:1) and/or 5 ng/ml recombinant human IL-12 p70 (R&D Systems, Inc.) for 7 d. Doses of recombinant human IL-12 ranging from 0.1 to 10,000 pg/ml were used to de-

termine the dose–response. Supernatants were harvested after 2 d for quantification of TNF-α by ELISA and after 7 d for quantification of IFN-γ by ELISA (R&D Systems, Inc.). Alternatively, PBMC were stimulated with PHA and/or supernatants of EBV-B cells from the patient (transfected with p40 wt, p40 del4.4, or control expression vectors) and IFN-γ was quantified in the supernatant after 3 d. To obtain dendritic cells, adherent PBMC were cultured for 9 d in RPMI 1640 medium supplemented with 10% FCS (GIBCO BRL), 1,000 IU/ml IL-4 (Genzyme Corp., Cambridge, MA), and 1,600 IU/ml GM-CSF (Sandoz, Basel, Switzerland). Dendritic cells were activated for 40 h with recombinant soluble endotoxin-free CD40-ligand (a gift of Drs. Graber and Bonnefoy, Glaxo, Geneva, Switzerland). IL-12 p70, IL-12 p40, and IL-8 were quantified in the supernatant by ELISA (R&D Systems, Inc.).

Results

Results of routine immunologic investigation were normal in this child, ruling out classical immunodeficiencies as the cause of BCG infection (not shown). The diagnosis of IFN-γR1 deficiency was excluded on the basis of flow cytometry with specific antibodies, gene sequencing, and cellular responses to IFN-γ which also excluded defects in genes encoding proteins associated with IFN-γR1, such as IFN-γR2 (not shown). A major defect in IFN-γ was considered unlikely given the normal detection of the cytokine in the supernatant of PHA-activated PBMC with several specific antibodies (not shown).

The secretion of IFN-γ by lymphocytes was quantified after stimulation of PBMC with BCG. Induction of IFN-γ was markedly impaired in the patient when compared with a control (Fig. 2 *A*). However, addition of recombinant exogenous IL-12 p70 in the assay was able to restore normal IFN-γ production. Likewise, IL-12 alone was sufficient to induce high levels of IFN-γ production. A dose-dependent response was further demonstrated (Fig. 2 *B*). This suggested that impaired IFN-γ secretion by the patient's cells is a consequence of insufficient IL-12 production rather than a consequence of an intrinsic IFN-γ gene defect or of a defective response to IL-12.

We thus investigated the role of IL-12 p70, a potent IFN-γ–inducing heterodimeric cytokine (composed of p40 and p35 subunits) secreted by phagocytes and dendritic cells (14). After amplification by PCR, sequencing of the IL-12 p40 subunit cDNA in the patient revealed a frameshift deletion of 373 nucleotides between positions 482 and 854. By Northern blot, the IL-12 p40 transcript was expressed in approximately normal amounts in PMA-stimulated EBV-transformed B cells (not

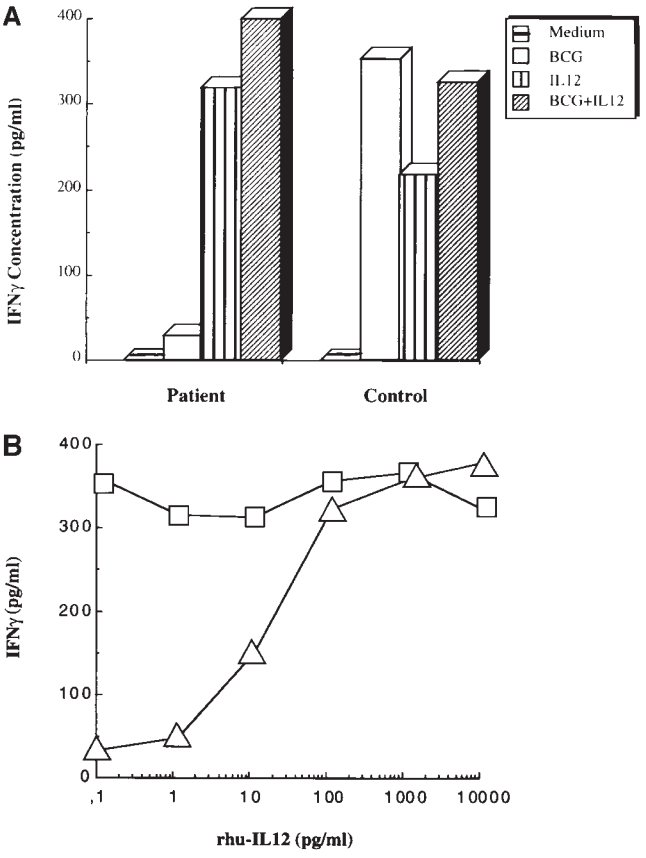

Figure 2. Impaired IFN-γ production and complementation by addition of exogenous IL-12. (*A*) Production of IFN-γ by PBMC stimulated with BCG and/or exogenous recombinant IL-12 p70 (5 ng/ml). Supernatants were harvested after 7 d for quantification of IFN-γ by ELISA. (*B*) IL-12 dose-dependent complementation of IFN-γ induction; the same experiment was repeated with various concentrations of recombinant human IL-12 p70 in the patient (*triangles*) and a healthy individual (*squares*). This experiment is representative of two separate experiments.

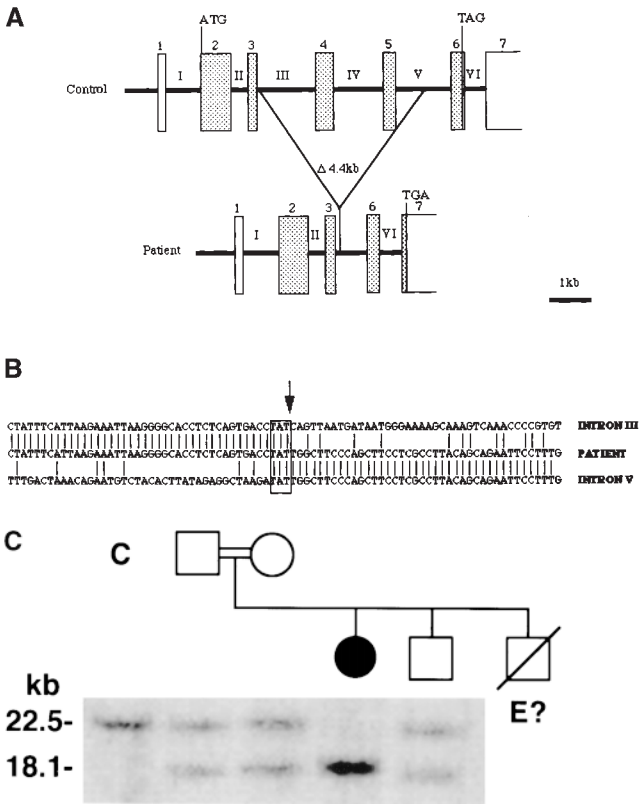

Figure 3. A large homozygous deletion within the *IL12P40* gene. (*A*) Genomic structure of the *IL12P40* gene and deletion in the patient. (*B*) Recombination breakpoints (*arrow*) and sequence motif upstream of the breakpoints (*boxed*). (*C*) Pedigree and intrafamilial segregation of the *IL12P40* genomic deletion compared with a control (*C*); no material was available from a deceased sibling (Bennett et al. [25]).

shown). No mutation was found in the IL-12 p35 subunit cDNA. The gene encoding human IL-12 p40 (designated as *IL12P40*) was shown to consist of at least 7 exons, and a deletion of 4.4 kb encompassing two coding exons was found in the patient (designated as del4.4) (Fig. 3 *A*). This deletion was not found in 30 unrelated healthy individuals investigated. Three nucleotides adjacent to the two recombination breakpoints were identical and may have contributed to the recombination process (Fig. 3 *B*). The parents and the healthy sibling were heterozygous for the deletion, whereas the patient was a homozygote, as detected by sequencing of genomic PCR products (not shown) and Southern blot (Fig. 3 *C*).

Both IL-12 p70 (10–15 pg/ml) and its p40 subunit (200–250 pg/ml) were detected by ELISA in the supernatant of BCG-activated control PBMC. Likewise, p70 (60–80 pg/ml) and p40 (15,000–20,000 pg/ml) were secreted by soluble CD40L-activated control dendritic cells derived in vitro. In contrast, neither p70 nor p40 was detected in the supernatant of the patient's cells, whereas induction of TNF-α and IL-8 confirmed normal activation of phagocytes and dendritic cells, respectively (Fig. 4 *A*). The wild-type mature IL-12 p40 protein consists of 307 amino acids, whereas the mutant IL-12 p40 protein, due to the genomic deletion and the secondary frameshift in the coding region, consists of 184 amino acids including only 139 original amino acids in the NH_2-terminal region and 45 novel amino acids in the COOH-terminal region. It is not known whether the mutant IL-12 p40 polypeptide is stable, in which case the lack of detection by specific antibodies may reflect altered epitopes.

To ascertain that homozygous *IL12P40* del4.4 is responsible for the lack of detectable IL-12 p40 and p70, an EBV-transformed B cell line from the patient was transfected with either *IL12P40* wild-type cDNA (p40wt), *IL12P40* del4.4 cDNA (p40del4.4) cDNA, or control expression vector. Values of IL-12 p40 obtained with the patient's cells transfected with p40wt were similar to those of control cells, whereas no p40 was detected in the supernatant of the patient's untransfected cells, and of those transfected with p40del4.4 cDNA or control vector (Fig. 4 *B*). Secreted p70 was also detected after transfection of the patient's cells with p40wt. Moreover, IL-12 in this supernatant was biologically active, because much higher levels of IFN-γ were secreted by the patient's PBMC stimulated with PHA and the supernatant, when compared with PHA stimulation with or without the other supernatants (not shown). These results demonstrate that there is a causative relationship between homozygous *IL12P40* del4.4 mutation and impaired secretion of functional IL-12 in the patient.

Discussion

Herein, we have reported the first human disease due to a cytokine gene defect. Autosomal recessive IL-12 deficiency, caused by a large *IL12P40* gene deletion encompassing two coding exons, is associated in our patient with BCG and *S. enteritidis* disseminated infection. Another kindred with impaired IL-12 production (of hitherto unknown molecular basis) in patients with *Mycobacterium avium* infection has also been reported (15). Its pedigree is more compatible with X-linked than autosomal recessive inheritance, suggesting that there may be another genetic defect leading to impaired IL-12 production. Recently, other patients with infections due to BCG, nontuberculous mycobacteria, and salmonella were found to be homozygous for null mutations in the gene encoding the IL-12Rβ1 chain (12, 13). Together, these studies sug-

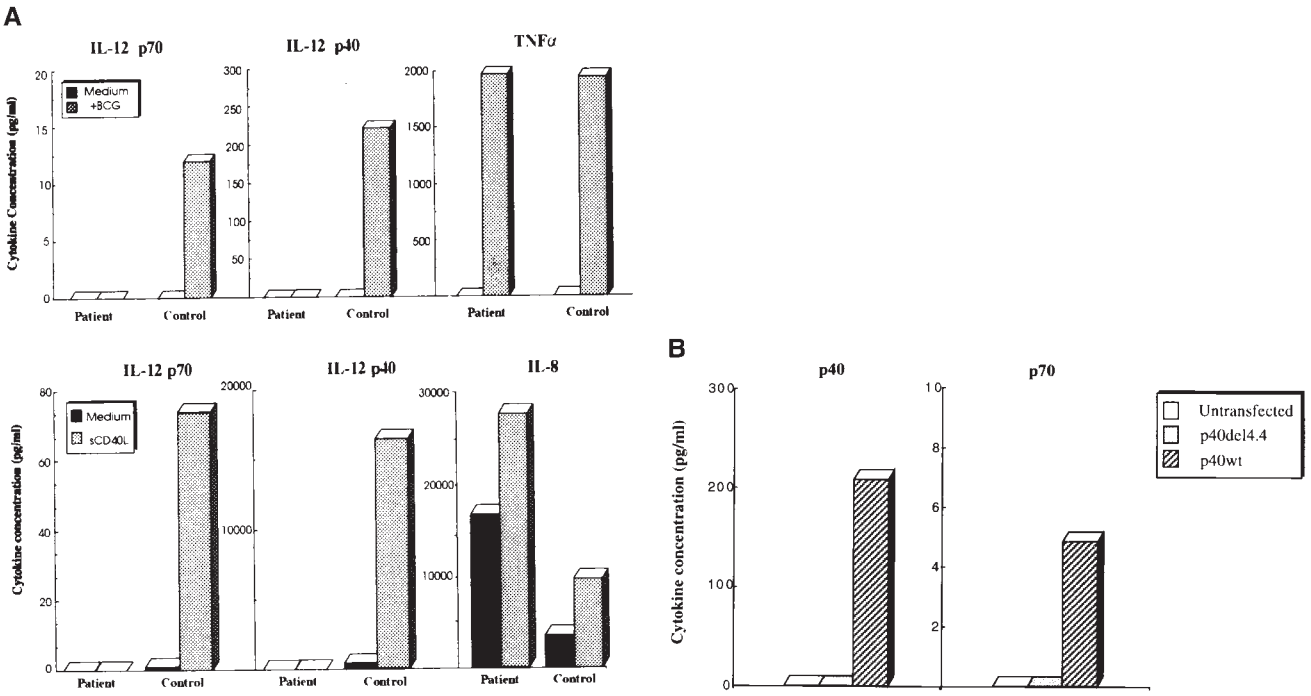

Figure 4. Impaired IL-12 production and complementation by transfection with wild-type *IL12P40* gene. (*A*) Impaired IL-12 p40 and p70 secretion by BCG-stimulated PBMC (*top*) and CD40L-stimulated dendritic cells (*bottom*). (*B*) Impaired IL-12 p40 and p70 secretion by an EBV-transformed B cell line from the patient is complemented by transfection with wild-type *IL12P40* cDNA (p40wt) only. Each of these experiments is representative of three distinct experiments.

gest that there is a cause and effect relationship between impaired IL-12–mediated immunity and vulnerability to infections due to poorly virulent mycobacteria and salmonella.

Studies in the mouse also support this conclusion. Mice with disrupted *IL12P40* gene (16) are highly susceptible to *Mycobacterium tuberculosis* (17), *M. bovis* BCG (18), and *M. avium* (19). A growing body of experimental evidence in humans and in mice further suggests that IL-12 is important for the control of a wide range of viral, prokaryotic, and eukaryotic microorganisms (20). However, the experiment of Nature herein reported together with previous observations on IL-12 receptor–deficient patients rather suggest that IL-12 is not necessary for the control of most microbes, including surprisingly a number of intracellular pathogens other than mycobacteria and salmonella. More patients need to be investigated to better appreciate the full range of potential pathogens.

The role of human IL-12 in defense against mycobacteria and salmonella cannot be compensated for by other immune interactions in vivo. Interestingly, the occurrence of severe infections due to the same microbial species in IFN-γR1–deficient children showed previously that IFN-γ is irreplaceable to control mycobacteria and salmonella (for reviews see references 21–24). Even though IL-12 is secreted by macrophages and dendritic cells and IFN-γ by NK and T lymphocytes, both cytokines appear to be essential for mycobacterial immunity in humans. Children genetically deprived of IL-12–mediated immunity, due to either IL-12P p40 or IL-12Rβ1 defect, have impaired IFN-γ production by NK and T lymphocytes in vitro. Moreover, IFN-γ therapy appears to be beneficial in vivo, as attested by the marked symptomatic improvement after cytokine therapy was commenced in IL-12 p40– and IL-12Rβ1–deficient patients. These observations suggest that the susceptibility to mycobacterial infection of patients with genetically impaired IL-12–mediated immunity is due to insufficient IFN-γ–mediated immunity.

Along these lines, it is likely that residual (IL-12–independent) IFN-γ secretion accounts for the milder clinical phenotype of IL-12 p40– and IL-12Rβ1–deficient patients, when compared with patients with complete IFN-γR1 or IFN-γR2 deficiency. In the patient with IL-12 p40 deficiency, like in IL-12Rβ1–deficient patients, mature BCG granulomas were seen, confirming that the formation of tuberculoid BCG granulomas is not strictly IL-12–dependent (whereas the lack of mature granulomas in all patients with IFN-γR1 or IFN-γR2 deficiency showed that their formation is strictly IFN-γ–dependent). However, the occurrence of mature granulomas, presumed to restrict microbial growth, in individuals with disseminated BCG infection, is paradoxical. Our observation that the development of mature granulomas appeared to be delayed, since only poorly circumscribed and differentiated multibacillary lesions were seen at early stages of BCG infection, probably resolves this paradox.

It is intriguing that the father of the child with homozygous IL-12 deficiency had severe persistent systemic infection with a nontyphi strain of salmonella (*S. bareilly*) and even more intriguing that the grandmother had pulmonary tuberculosis. It can be expected that other IL-12–deficient patients (homozygotes or compound heterozygotes for null mutations) may be susceptible to poorly pathogenic salmonella and mycobacteria, and it is also likely (albeit not proven to date) that such patients may be susceptible to *M. tuberculosis*. It can be further speculated that heterozygotes for the *IL12P40* deletion may be intrinsically more vulnerable to poorly virulent species, such as *S. bareilly*, and to more virulent species, such as *M. tuberculosis*. In view of the kindred that we document here, further studies are warranted to test this hypothesis.

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