

## Brief Report

**INTERFERON- $\gamma$ -RECEPTOR  
DEFICIENCY IN AN INFANT WITH  
FATAL BACILLE CALMETTE-GUÉRIN  
INFECTION**

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**T**HE attenuated strain of *Mycobacterium bovis* bacille Calmette-Guérin (BCG) is the most widely used vaccine in the world. In most children, inoculation of live BCG vaccine is harmless although it occasionally leads to a benign regional adenitis.<sup>1</sup> In rare cases, however, vaccination causes disseminated BCG infection, which may be lethal. Impaired immunity of the host is generally thought to be the pathogenic mechanism. Disseminated BCG infection has been reported in children with inherited immune disorders. Most of these children had severe combined immunodeficiency, which is characterized by an absence of T cells, and some had chronic granulomatous disease, which is marked by an impairment of the phagocyte respiratory burst.<sup>2,3</sup> Rare cases of BCG infection have also been reported in association with the acquired immunodeficiency syndrome.<sup>2</sup>

However, a specific immunodeficiency can be identified in only about half the cases of disseminated BCG infection.<sup>2</sup> In the other cases, the pathogenesis remains unclear. Such idiopathic cases have been reported in 24 countries, with a prevalence in France of at least 0.59 case per 1 million children vaccinated with BCG.<sup>4</sup> The high rates of consanguinity (30 percent) and familial forms (17 percent) and the

equal sex distribution support the hypothesis of a new type of primary immune disorder with an autosomal recessive pattern of inheritance.

The pathological features and clinical outcomes suggest that there are two distinct forms of idiopathic BCG infection and a genetic heterogeneity of the underlying immune disorder.<sup>5</sup> Well-circumscribed and well-differentiated tuberculoid granulomas with few visible acid-fast rods are associated with a good prognosis. In contrast, ill-defined and poorly differentiated, leproma-like granulomas with many visible bacilli are associated with a fatal outcome, despite antimycobacterial therapy. We reasoned that the latter form of idiopathic BCG infection most likely results from a genetic defect affecting an obligatory and relatively specific step in the formation of a bactericidal BCG granuloma.

Mice in which the gene for the interferon- $\gamma$ -receptor high-affinity binding chain (interferon- $\gamma$ R1) has been deleted are highly susceptible to BCG infection, with defects in granuloma structure and a fatal outcome.<sup>6</sup> In mice with deletions of the interferon- $\gamma$  gene or the gene for interferon- $\gamma$  regulatory factor 1 (IRF1), there is a failure to control BCG growth.<sup>7,8</sup> Mice treated with antibodies against tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) are susceptible to BCG infection, with defective granuloma structure and a fatal outcome.<sup>9</sup> Such a dramatic effect is not observed, however, in mice with deletions of the gene for TNF- $\alpha$  receptor 1 (TNF- $\alpha$ R1).<sup>10</sup>

We examined the five genes coding for interferon- $\gamma$ , interferon- $\gamma$ R1, IRF1, TNF- $\alpha$ , and TNF- $\alpha$ R1 in a child with fatal idiopathic disseminated BCG infection. We found a mutation of the gene for interferon- $\gamma$ R1. There was no detectable interferon- $\gamma$ R1 on the cells from the affected child. These findings provide further evidence of the importance of interferon- $\gamma$  in the response to mycobacterial infection.

#### CASE REPORT

A girl was born at term to parents of Tunisian origin who were first cousins (Patient 16 in Casanova et al.<sup>4</sup>). Her size and weight were normal, and she had no overt developmental defects. She was vaccinated with 25 mg of BCG substrain Pasteur at one month of age. Two older brothers, previously vaccinated with BCG, were healthy. She was healthy until 2.5 months of age, when fever and regional adenitis developed. Cachexia, granulomatous dermatitis, hepatosplenomegaly, lymph-node enlargement, diffuse pneumonitis, and multiple osteolytic lesions developed rapidly, with continued fever. The values for blood leukocytes, erythrocyte sedimentation rate, serum C-reactive protein, and serum fibrinogen were elevated. Lymph-node and skin biopsies revealed ill-circumscribed areas of macrophages filled with acid-fast bacilli (Fig. 1). There were no epithelioid or giant cells or surrounding lymphocytes within these leproma-like granulomas. Treatment with antimycobacterial agents (including rifampin, isoniazid, ethambutol, streptomycin, and clofazimine) was initiated. One month later, a mycobacterium species was cultured from the lungs and bone marrow and identified as *M. bovis* BCG strain.

Despite antimycobacterial treatment and adjuvant treatment with interferon gamma, the child died at the age of 10 months from BCG infection with multiorgan failure, including bone mar-

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row and liver failure. No other opportunistic infectious agents were identified. Neither autoimmune nor allergic processes were noted. A diagnosis of idiopathic disseminated BCG infection was made on the basis of the absence of clinical, radiologic, or biologic signs of any known immunodeficiency.<sup>3</sup> In particular, the spleen and lymph nodes were grossly normal. Immunologic studies showed a normal leukocyte count, including normal proportions of polymorphonuclear cells, B cells, CD4 and CD8 T cells,  $\alpha/\beta$  and  $\gamma/\delta$  T cells, and natural killer cells. IgM, IgG, IgA, and IgE levels were within the normal ranges or elevated. Complement levels were within the normal ranges. Lymphocyte-function-associated antigen 1 and HLA class I and II molecules were present on the cell surface. The reduction of nitroblue tetrazolium by neutrophilic polymorphonuclear leukocytes was normal. Mitogen-driven and tuberculin-driven T-cell proliferations were normal. A test of delayed hypersensitivity to purified protein derivative (10 IU) was positive (a 16-mm induration). Antimycobacterial serologic studies were also positive. Specific serologic tests for tetanus toxoid and poliovirus were positive after immunization. Repeated serologic tests for infection with the human immunodeficiency virus were negative. All further investigations, reported below, were performed on a B-cell line transformed by Epstein-Barr virus (EBV).

## METHODS

### Microsatellites

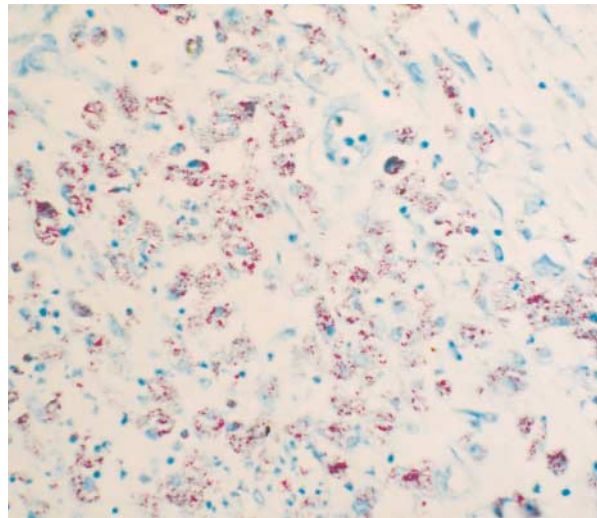
The genes coding for human TNF- $\alpha$ , TNF- $\alpha$ R1, interferon- $\gamma$  (IFN- $\gamma$ ), and IRF1 have been mapped to 6p21.3, 12p13, 12q24, and 5q31.1, respectively. Polymorphic microsatellites within a 2-cM interval around each of these genes, available from human-genome data bases, included TNF1 (for TNF- $\alpha$ ), D12S93 and D12S314 (for TNF- $\alpha$ R1), D12S342 (for IFN- $\gamma$ ), and D5S393 (for IRF1). The IFN- $\gamma$ R1 gene was mapped at the Genethon Laboratories (Evry, France) with the use of irradiation hybrids within a larger (10 cM) interval (D6S262 through D6S293) on 6q21-q23.

### Sequencing of Complementary DNA

Total RNA was extracted from EBV-transformed B cells in guanidium isothiocyanate, as described elsewhere.<sup>11</sup> Complementary DNA (cDNA) was synthesized by incubating 10  $\mu$ g of total RNA with oligo-dT<sub>12-18</sub> and avian myeloblastosis virus reverse transcriptase according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Polymerase chain reaction (PCR) with IFN- $\gamma$ R1 sense (5'TTAAGCTTGGAGCCAGCGACCGT3') and antisense (5'CGGATCCAAAGTTGGTGCAACT3') primers<sup>12</sup> was carried out with a mixture of *Taq* polymerase (Promega, Madison, Wis.) and Pfu polymerase (Stratagene, La Jolla, Calif.) under the following conditions: five minutes at 94°C, followed by 35 cycles, each for one minute at 94°C, two minutes at 55°C, and three minutes at 72°C. PCR products were treated with *Taq* polymerase and deoxyadenosine triphosphate for 60 minutes at 72°C, ligated to pGEM-T vectors (Promega), and transformed into JM109 bacteria. Recombinant phagemids were sequenced with Sequenase or Thermosequenase (U.S. Biochemical, Cleveland) with the use of a series of nested primers. Double-stranded PCR products were directly sequenced on both strands around nucleotide 131 (nucleotides were numbered starting with the ATG sequence initiating the coding region) with Sequenase<sup>13</sup> or Thermosequenase.

### Genomic PCR

Genomic DNA was extracted from EBV-transformed B cells with proteinase K, sodium dodecyl sulfate, and a series of phenol-chloroform extractions.<sup>11</sup> Genomic sense (5'GCCTACACCAACTAATGTTA3') and antisense (5'ATAGTTCTTTACCTCTACGG3') primers within exon 2 of the IFN- $\gamma$ R1 gene (Merlin G, et al.: personal communication) were used for genomic PCR. Genomic PCR was performed with the incorporation of [ $\alpha$ <sup>32</sup>P]deoxycytidine triphosphate and analyzed on a sequencing gel.



**Figure 1.** Leproma-like Granuloma in a Lymph-Node-Biopsy Specimen from an Infant with Bacille Calmette-Guérin Infection. The granuloma has ill-circumscribed areas of poorly differentiated macrophages filled with acid-fast bacilli (Ziehl stain,  $\times 400$ ).

### Northern Blotting

Total RNA (10  $\mu$ g) from EBV-transformed B cells was run on a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a <sup>32</sup>P-labeled double-stranded DNA probe corresponding to the IFN- $\gamma$ R1 PCR product or to an actin fragment.<sup>11</sup>

### Assay of <sup>125</sup>I-Labeled-Interferon- $\gamma$ Binding

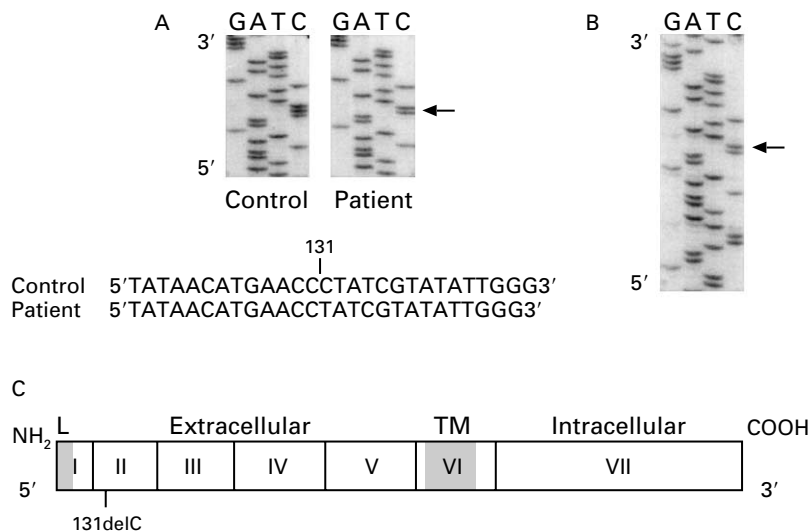
One million EBV-transformed B cells were incubated for two hours at 4°C in RPMI 1640 supplemented with 5 percent fetal-calf serum in the presence of various amounts of <sup>125</sup>I-labeled interferon- $\gamma$  (specific radioactivity,  $50 \times 10^6$  cpm per microgram). Parallel samples were incubated under the same conditions with a 100-fold excess of unlabeled interferon- $\gamma$ . Cell suspensions were washed four times with phosphate-buffered saline containing 1 percent fetal-calf serum, 0.1 mM calcium chloride, and 0.1 mM magnesium chloride, and the radioactivity of the cell pellet was counted. Specific binding was calculated as the bound radioactivity that could be displaced by cold interferon- $\gamma$  (the mean value of triplicate measurements).

### Cell Staining

The available murine monoclonal antibodies specific for human interferon- $\gamma$ R1 were 5362-6545 (IgG1) (Valbiotech, Paris) and 1224-00 (IgG2b) (Genzyme, Cambridge, Mass.). Cells ( $2 \times 10^5$ ) were incubated first in RPMI containing 10 percent AB+ serum with 2  $\mu$ g of ad hoc monoclonal antibodies, then with a biotinylated goat antimouse antibody (Immunotech, Marseille, France), and finally with streptavidin-phycoerythrin (Tebu, Paris). Staining was detected by flow cytometry (Becton Dickinson, Oxford, United Kingdom). Specific staining of interferon- $\gamma$ R1 on EBV-transformed B cells was deduced from that obtained with an isotopic control antibody.

## RESULTS

We first analyzed the intrafamilial segregation of polymorphic microsatellites located near each of the five candidate genes coding for TNF- $\alpha$ , TNF- $\alpha$ R1,



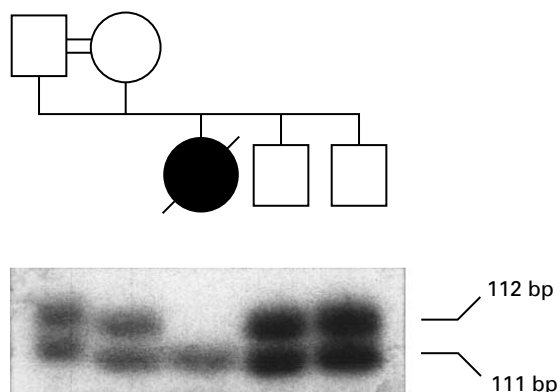
**Figure 2.** Mutation of the Messenger RNA Coding Region of the *Interferon- $\gamma$ R1* Gene.

Panel A shows the results of a sequence analysis of reverse-transcriptase-polymerase-chain-reaction (PCR) products around nucleotide 131 (numbered from the ATG sequence initiating the coding region) from the patient and a control. The arrowhead indicates the deletion of nucleotide 131 in the patient. Panel B shows the results of direct sequencing of an independent reverse-transcriptase-PCR product from the patient in the same region. Panel C shows a schematic representation of *interferon- $\gamma$ R1* messenger RNA (mRNA) with exon boundaries and protein with domain boundaries. Hydrophobic regions are shaded. L denotes leader domain and TM transmembrane domain. The deletion of nucleotide 131 results in a frame shift and a premature stop codon at nucleotides 187 through 189 in exon 2.

*interferon- $\gamma$* , *interferon- $\gamma$ R1*, and *IRF1*. Since the child had been born to consanguineous parents, she was most likely homozygous for a mutant allele inherited from a common ancestor. Thus, we reasoned that microsatellite heterozygosity would rule out nearby genes, and homozygosity would prompt further investigation. Intrafamilial segregation of microsatellites suggested that only the gene coding for *interferon- $\gamma$ R1* warranted further investigation (data not shown).

*Interferon- $\gamma$ R1* is ubiquitously expressed, and the cDNA coding region could therefore be amplified and sequenced from an EBV-transformed B-cell line. Two recombinant phagemids from distinct PCR products lacked nucleotide 131 in the coding region (Fig. 2A).<sup>12</sup> No other mutations were found. The single-base deletion was confirmed by direct sequencing of an independent PCR product (Fig. 2B). The single-nucleotide deletion designated 131delC creates a frame shift and leads to a premature stop codon (TAA) at nucleotides 187 through 189 (Fig. 2C). Both the deletion and the stop codon are located in a region that codes for the N-terminal portion of the extracellular domain of the receptor.

Intrafamilial segregation of the mutant allele of the *IFN- $\gamma$ R1* gene was analyzed to confirm its role in the pathogenic process. On the basis of the genomic structure of *IFN- $\gamma$ R1* (Merlin G, Dembic Z:



**Figure 3.** Intrafamilial Segregation of the Mutation in the *Interferon- $\gamma$ R1* Gene.

The results of sequencing gel analysis of radioactive PCR products are shown for genomic DNA from the patient and her father, mother, and healthy brothers, with genomic sense and antisense primers specific for exon 2 of the *interferon- $\gamma$ R1* gene. The normal product is 112 bp, and the mutant product is 111 bp. The squares denote male family members, and the circles female family members; the solid circle with a slash denotes the dead patient.

personal communication), the mutation was determined to be located in exon 2 (Fig. 2C). A genomic PCR product around the single-base deletion within exon 2 showed that the affected child carried the mutant allele at both *IFN- $\gamma$ R1* loci (Fig. 3). Both her parents and her two healthy brothers were heterozygous carriers.

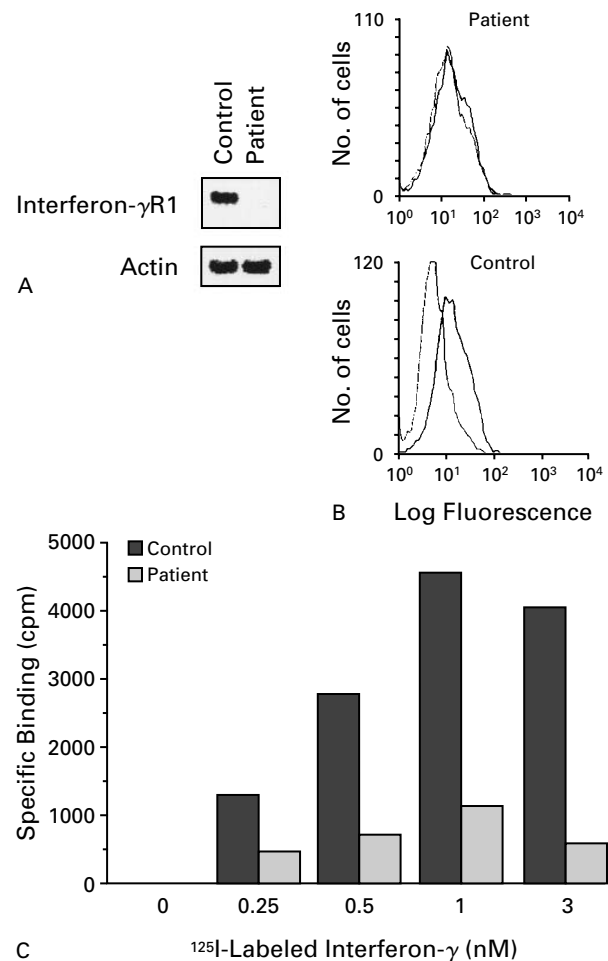
Northern blot analysis revealed a considerable decrease in detectable interferon- $\gamma$ R1 messenger RNA (mRNA) in EBV-transformed B cells from the affected child, presumably due to a rapid degradation of the transcript (Fig. 4A). Hybridization of the same blot with a control probe (actin) revealed no difference in size or intensity between mRNA from a control and mRNA from the patient. The 2.3-kb specific transcript, along with a degradation smear, was visible with longer exposure (data not shown).

We analyzed the expression of interferon- $\gamma$ R1 on the EBV-transformed B-cell surface by flow cytometry with two specific monoclonal antibodies (Fig. 4B). The specific binding of two monoclonal antibodies, each expressing a distinct isotype, on a control cell line was faint. The expression of cell-surface interferon- $\gamma$ R1 on the cell line from the affected child, however, was not detectable on repeated experiments with either monoclonal antibody.

Specific binding of  $^{125}$ I-labeled interferon- $\gamma$  was determined on EBV-transformed B cells from a control and the patient to confirm the absence of an interferon- $\gamma$ R1 binding site for interferon- $\gamma$  on the cell surface (Fig. 4C). As expected, there was dose-dependent specific interferon- $\gamma$  binding in the control cell line. In contrast, there was dramatically reduced specific binding in the patient's cell line. Scatchard analysis revealed high-affinity (association constant,  $1.25 \times 10^{-9}$  M) specific binding on approximately 6500 binding sites per cell in the control line. The residual specific binding in the patient's cell line did not allow a precise Scatchard analysis. Altogether, our data show a complete deficiency of cell-surface expression of interferon- $\gamma$ R1 in the child.

## DISCUSSION

In this child with a fatal BCG infection and in a kindred with atypical mycobacterial infection described by Newport et al. in this issue of the *Journal*,<sup>14</sup> similar, yet distinct, mutations of the *IFN- $\gamma$ R1* gene were found that precluded the expression of interferon- $\gamma$ R1. It thus appears that a complete deficiency of interferon- $\gamma$ R1 may lead to BCG infection in vaccinated children or to atypical mycobacterial infection in unvaccinated persons. The prognosis appears to be worst after BCG vaccination, probably because of the higher virulence of BCG as compared with most atypical mycobacteria, the higher number of infecting BCG organisms, or the direct intradermal inoculation of BCG as opposed to natural routes of infection with atypical mycobacteria. In any event,



**Figure 4.** Results of Studies of Cell-Surface Interferon- $\gamma$  Receptor (Interferon- $\gamma$ R1) in the Patient and a Control.

Panel A shows the results of a Northern blot analysis of interferon- $\gamma$ R1 and actin transcripts from Epstein-Barr virus (EBV)-transformed B cells. Panel B shows the staining of EBV-transformed B cells with an IgG1 monoclonal antibody specific for interferon- $\gamma$ R1, analyzed by flow cytometry. The dashed lines indicate the values obtained with an isotypic control antibody. Panel C shows the specific binding of  $^{125}$ I-labeled interferon- $\gamma$  to EBV-transformed B cells.

vaccination with all currently used live BCG substrains is contraindicated in children with interferon- $\gamma$ R deficiency. Whether new generations of live vaccine, such as auxotrophic BCG substrains,<sup>15</sup> are safe and even protective in such children is unknown.

Fatal disseminated BCG or atypical mycobacterial infections in other patients may be due to a complete deficiency of interferon- $\gamma$ R1. Milder forms of idiopathic disseminated BCG infections, characterized by well-circumscribed tuberculoid granulomas and few visible acid-fast rods within macrophages, have a favorable outcome<sup>5</sup> and may represent either different mutations of the *IFN- $\gamma$ R1* gene or muta-

tion of another gene. Furthermore, alternative or heterozygous mutations of the *IFN- $\gamma$ R1* gene may contribute to the genetic susceptibility to more virulent mycobacteria in tuberculosis<sup>16</sup> or leprosy.<sup>17</sup> Mutations of the *IFN- $\gamma$ R1* gene may also confer a predisposition to other infections with intracellular microorganisms, such as salmonella, which are frequently associated with idiopathic BCG or atypical mycobacterial infections.<sup>4</sup> In any event, a complete deficiency of interferon- $\gamma$ R1 provides molecular evidence of a selective genetic susceptibility to mycobacterial infection.

From recent studies in mice, much has been learned about the characteristics of interferon- $\gamma$  at the cellular level.<sup>6-8,18-20</sup> After macrophage stimulation, such as that produced by mycobacterial infection, the secretion of TNF- $\alpha$ , interleukin-12, and possibly other factors by macrophages promotes interferon- $\gamma$  secretion by natural killer cells, differentiation of antigen-driven CD4 T cells into interferon- $\gamma$ -producing TH1 cells, and activation of these TH1 cells to secrete interferon- $\gamma$  and possibly other macrophage-activating factors.<sup>18,19</sup> Secretion of interferon- $\gamma$ , in turn, results in macrophage secretion of TNF- $\alpha$ <sup>9</sup>; activation of macrophage mycobactericidal mechanisms, such as nitric oxide production<sup>8</sup>; and impaired proliferation of interleukin-4-secreting TH2 cells.<sup>20</sup>

It is likely that the susceptibility to mycobacterial infection in children with a complete deficiency of interferon- $\gamma$ R1 results primarily from an intrinsic impairment of macrophages rather than a defective TH1 response. The strongly positive test for delayed hypersensitivity to tuberculin in the child we studied reflects a normal TH1 response, which is similar to the findings in mice with deletions of the *IFN- $\gamma$ R1* gene.<sup>21</sup> The normal TH1 response may result from the fact that interferon- $\gamma$  does not directly promote TH1 responses but instead impairs TH2 responses.<sup>20</sup> The uncontrolled growth of mycobacteria within macrophages from interferon- $\gamma$ R1-deficient children is probably due to impaired activation of bactericidal mechanisms rather than impaired induction of major-histocompatibility-complex (MHC) class II molecules. Indeed, disseminated BCG infection after vaccination has not been reported in children with a complete deficiency of MHC class II molecules.<sup>2</sup> In vitro experiments in humans provide conflicting evidence, with interferon- $\gamma$  alternatively enhancing or reducing the growth of mycobacteria within cultured monocyte macrophages.<sup>22-28</sup> Moreover, the mycobactericidal mechanisms of human macrophages remain largely unknown.<sup>28</sup> Thus, it is likely that the macrophage has a causal role in the pathogenesis of mycobacterial infections associated with interferon- $\gamma$ R1 deficiency, but more direct and definitive evidence of such a role is required.

The selective susceptibility of children with a com-

plete deficiency of interferon- $\gamma$ R1 contrasts with the broad susceptibility of mice with deletions of the *IFN- $\gamma$*  or *IFN- $\gamma$ R1* gene, which are susceptible not only to mycobacterial infection,<sup>6,7,29,30</sup> but also to infection with other intracellular<sup>31</sup> or extracellular<sup>32</sup> bacteria, as well as a number of viruses<sup>31,33-35</sup> and parasites.<sup>21,36</sup> Children with interferon- $\gamma$ R1 deficiency do not appear to be susceptible to infection with agents other than mycobacteria. This apparently selective susceptibility may be the result of an underestimation of human interferon- $\gamma$ -mediated immunity due to early cases of fatal mycobacterial infection that preclude exposure to other potential pathogens; an overestimation of murine interferon- $\gamma$ -mediated immunity due to experimental, as opposed to natural, modes of infection; or intrinsic differences between human and murine interferon- $\gamma$ -mediated immunity.

The clinical features and outcome of interferon- $\gamma$ R1 deficiency in children show that interferon- $\gamma$  is obligatory for both an appropriate granuloma structure and efficient macrophage antimycobacterial activity. This condition thus adds weight to the growing evidence supporting the use of interferon- $\gamma$  as a therapeutic agent in patients with other mycobacterial diseases.<sup>37,38</sup>

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