A MUTATION IN THE INTERFERON-γ–RECEPTOR GENE AND SUSCEPTIBILITY TO MYCOBACTERIAL INFECTION

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ABSTRACT

Background Genetic differences in immune responses may affect susceptibility to mycobacterial infection, but no specific genes have been implicated in humans. We studied four children who had an unexplained genetic susceptibility to mycobacterial infection and who appeared to have inherited the same recessive mutation from a common ancestor.

Methods We used microsatellite analysis, immunofluorescence studies, and sequence analysis to study the affected patients, unaffected family members, and normal controls.

Results A genome search using microsatellite markers identified a region on chromosome 6q in which the affected children were all homozygous for eight markers. The gene for interferon-γ receptor 1 maps to this region. Immunofluorescence studies showed that the receptor was absent on leukocytes from the affected children. Sequence analysis of complementary DNA for the gene for interferon-γ receptor 1 revealed a point mutation at nucleotide 395 that introduces a stop codon and results in a truncated protein that lacks the transmembrane and cytoplasmic domains.

Conclusions Four children with severe mycobacterial infections had a mutation in the gene for interferon-γ receptor 1 that leads to the absence of receptors on cell surfaces and a functional defect in the up-regulation of tumor necrosis factor α by macrophages in response to interferon-γ. The interferon-γ pathway is important in the response to intracellular pathogens such as mycobacteria. (N Engl J Med 1996;335:1941-9.)

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THE World Health Organization has estimated that more than 1.7 billion persons are infected with *Mycobacterium tuberculosis* worldwide.[1,2] A puzzling feature of mycobacterial infection is that clinically evident disease occurs in only a small proportion of those who are infected. However, the mechanisms that distinguish a successful immune response, which contains the infection, from an ineffective response, which enables progressive disease to occur, remain poorly understood. Familial clustering, racial differences in incidence, and twin studies suggest that genetic factors have a role in susceptibility.[3-5] However, in contrast to the situation in mice, in which a number of single gene defects have been associated with susceptibility to intracellular pathogens, including mycobacteria,[6-10] no such genes have been identified in humans.

We have previously described a group of related children from a village in Malta who appear to have a familial immunologic defect predisposing them to infection with a range of mycobacteria.[11] Despite intensive treatment three of the four affected patients have died, and the survivor has persistent infection. Immunologic studies have shown that the affected children have defective production of tumor necrosis factor α (TNF-α) in response to endotoxin and a failure to up-regulate this cytokine in response to interferon-γ.[11]

It is likely that all four affected Maltese children have the same autosomal recessive disorder owing to a single mutation introduced by a common ancestor.[12] The mutation originally occurred on a single chromosome and is therefore associated with a particular haplotype inherited with the mutant allele. It is possible to identify the chromosomal location of a recessive gene in such families by searching for regions of the genome for which all the affected family members are homozygous by descent.[13,14] Candidate genes identified within the region of homology can then be analyzed for causative mutations. We have used this approach to identify the genetic defect underlying the increased susceptibility to mycobacterial infection in our patients.

METHODS

Patients

The characteristics of the patients have been described elsewhere.[11] Briefly, four children from the same small town in Malta presented with disseminated atypical mycobacterial infection in the absence of a recognized immunodeficiency. They all had fever, weight loss, hepatosplenomegaly, bone lesions, and an intense acute-phase response. Two of the affected children are brothers whose parents are second cousins, and the third is related to them as a fourth cousin through both sets of parents. The family pedigree has been described elsewhere,[11,12] and the numbering of patients and family members follows that of Newport et al.[12] The genetic link between these three children and the fourth child has not been determined, but they all come from the same town,

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where intermarriage between families is common. Each child was infected with a different species or strain of mycobacterium (*M. fortuitum, M. chelonei,* and two strains of *M. avium*), suggesting a defect in host immunity, and one child also had a prolonged episode of salmonellosis. It is therefore likely that the children all had the same recessively inherited defect. Three of the children have died as a result of their infections.

All family members agreed to participate in this study, which was approved by the hospitals' ethics committees.

Microsatellite Analysis

DNA was prepared from peripheral-blood lymphocytes according to standard methods.[15] A set of polymorphic microsatellites derived from both the Genethon catalogue and the Cooperative Human Linkage Center were typed in the parents and three of the affected children. Amplification was performed with the polymerase chain reaction (PCR) according to standard Genethon conditions.[16] Amplified products were resolved on 6 percent polyacrylamide sequencing gels. The subjects were subsequently typed for additional markers in the region of homozygosity.

Immunofluorescence Studies of Peripheral-Blood Leukocytes

Peripheral-blood mononuclear cells were prepared by Ficoll–Hypaque (Nycomed, Oslo, Norway) gradient centrifugation. Granulocytes were isolated from the erythrocyte pellet by lysis of red cells with 155 mM ammonium chloride. The cells were incubated for 30 to 60 minutes on ice with 2 to 5 μg of an isotype control per milliliter (Becton Dickinson, Oxford, United Kingdom) or 2 to 5 μg of antibodies against interferon-γ receptor 1 per milliliter (mouse IgG2b [1224-00] or mouse IgG1 [1223-01]; Genzyme, Cambridge, Mass.). Both antibodies are directed against the extracellular domain. Bound antibody was detected with goat antimouse IgG antibody labeled with fluorescein isothiocyanate (Dako, Teddington, United Kingdom). A minimum of 3000 cells was analyzed for each test with an Epics Profile II cell analyzer (Coulter Electronics, Luton, United Kingdom). For the analysis of up-regulation of the receptor, lymphocytes were cultured for 24 hours in the presence or absence of 400 nM dexamethasone (Sigma Chemical, St. Louis).[17]

Sequence Analysis of Complementary DNA for the Gene for Interferon-γ Receptor 1

In three of the affected patients and an unrelated control, messenger RNA (mRNA) was extracted with the Microfast Track kit (Invitrogen, San Diego, Calif.) from 1 million lymphocytes after transformation with the Epstein–Barr virus (EBV). Reverse transcription was accomplished with a complementary DNA (cDNA) cycle kit (Invitrogen), and the open reading frame of the gene for interferon-γ receptor 1 was amplified by PCR with the primers 5′CCAGCGACCGTCGGTAGCAGC3′ and 5′ATCCTCTTTACGCTTTCAT3′, designed from published sequence data.[18] The PCR products were cloned with the TA (Invitrogen) cloning kit. One to five clones from separate PCR reactions from each subject were sequenced with the Sequenase kit (Amersham, Amersham, United Kingdom) according to the manufacturer's instructions.

For the PCR assay of genomic DNA from members of the affected families, primers were designed (5′GTTAAAGCCAGGGTTGGACA3′ and 5′CATCTCGGCATACAGCAAATTCTTGT3′) to amplify a 70-bp fragment across the mutation site from genomic DNA. A single-base mismatch was introduced at the penultimate 3′ nucleotide of the reverse primer sequence (a substitution of G for C, shown underlined in the primer) in order to create a *Tsp*45I restriction site in normal DNA. This enzyme recognizes the sequence GTCAC (or GTGAC). The normal genomic sequence is GTCAG for nucleotides 393 to 397; the mismatch in the reverse primer introduces a C at position 397, thus creating a *Tsp*45I site. However, in the mutant sequence, there is an A at position 395 and the restriction site is lost. PCR amplification was performed in a total volume of 30 μl containing 100 ng of DNA; 10 pM of each primer; 3.5 mM magnesium chloride; 75 mM potassium chloride; 10 mM TRIS (pH 9.2); 200 μM each of deoxyadenosine triphosphate, deoxycytosine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate; and 1 U of *Taq* polymerase at an annealing temperature of 55°C. Amplified products were digested with *Tsp*45I, resolved on 15 percent polyacrylamide gels, stained with ethidium bromide, and visualized under ultraviolet light.

Interferon-γ Responses

The ability of interferon-γ to up-regulate the production of TNF-α by monocytes was studied with an in vitro whole-blood assay, as described elsewhere.[11] The production of TNF-α in response to *Escherichia coli* lipopolysaccharide (1 μg per milliliter) was compared with TNF-α production induced by the same concentration of lipopolysaccharide after pretreatment with interferon-γ (2 μg per milliliter) for two hours. Plasma TNF-α levels were measured with an enzyme-linked immunosorbent assay.[19]

RESULTS

In an initial screening, 360 polymorphic microsatellite markers spaced at intervals of approximately 11 cM across the genome were typed in three of the affected children and their parents. Haplotype sharing on three or more of the six affected chromosomes was observed on a total of 42 chromosomal regions. These regions were further analyzed with additional microsatellite markers to determine whether they were identical by state or by descent. A single 5-cM region was identified on chromosome 6q in which all three affected children were homozygous for the same alleles for eight microsatellites, whereas their parents and unaffected siblings were not (Fig. 1). The gene for interferon-γ receptor 1 is located in this region[21] and seemed a likely candidate gene, since mice lacking this gene are susceptible to infections with mycobacteria.[7]

To establish whether the expression of interferon-γ receptor 1 was defective on the cells of the affected children, we investigated the binding of two monoclonal antibodies with specificities for different epitopes of the extracellular domain of the receptor. The antibodies did not bind to freshly isolated monocytes, neutrophils, or lymphocytes from the only surviving child, Subject III-4 (Fig. 2A, 2B, and 2C). The extent of binding to the cells from his mother (Subject II-4) was intermediate. Similarly, there was no binding to stored lymphocytes from Subject III-1 (Fig. 2D), whereas the extent of binding to his parents' cells (Subjects II-1 and II-2) was intermediate between that of the patients and that of the healthy controls (Fig. 2). The expression of other cell-surface proteins, including CD14, CD16, CD3, and the receptor for granulocyte–macrophage colony-stimulating factor, was normal (data not shown). Preincubation of fresh peripheral-blood mononuclear cells with dexamethasone resulted in the up-regulation of the receptor on lymphocytes from the control and an unaffected parent (Subject II-4), but not from the affected child (Subject III-4) (Fig. 2C).

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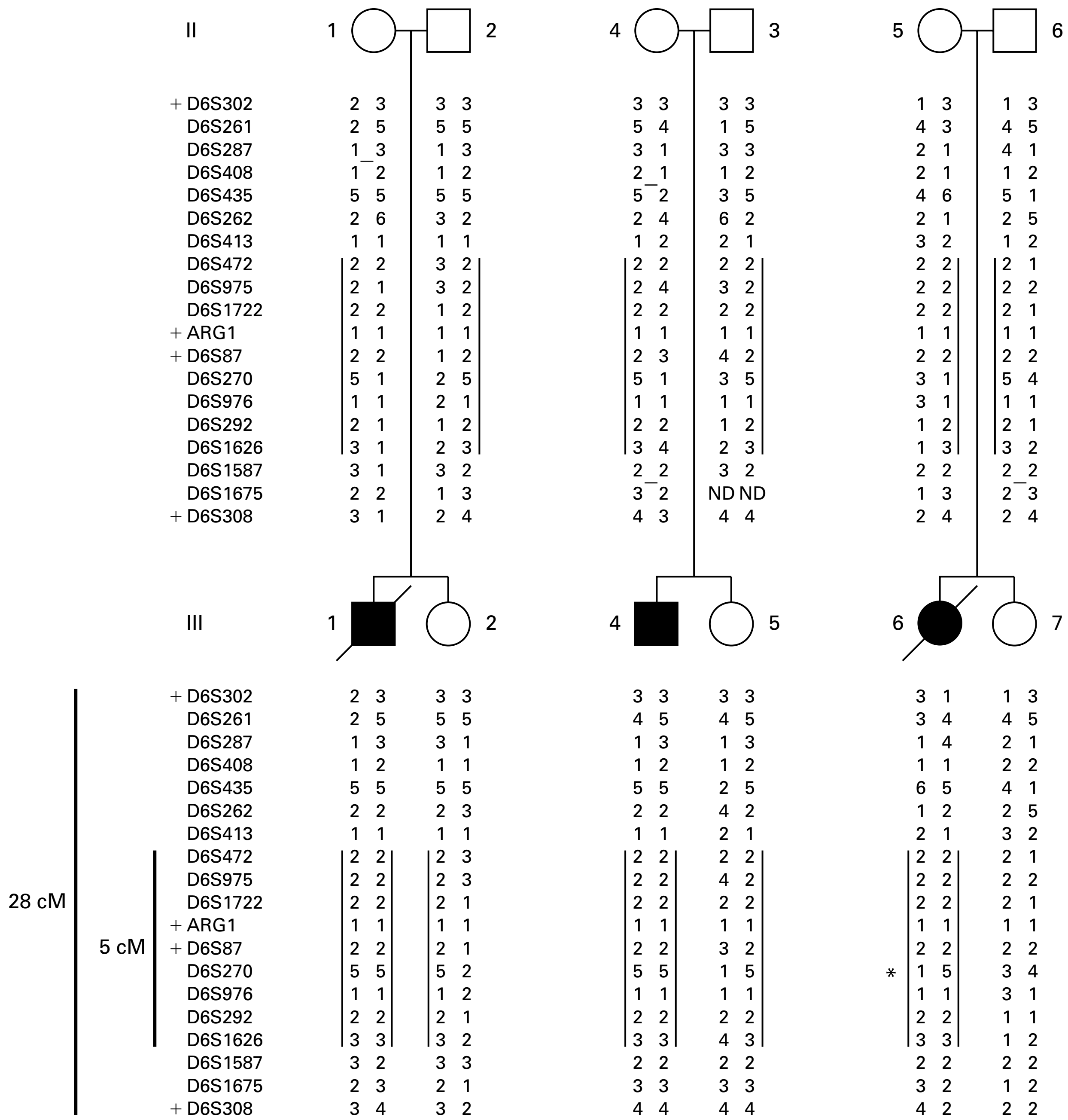

Figure 1. Haplotypes of Affected Children, Their Parents, and Unaffected Siblings in the Region of Chromosome 6q22–q23.

Squares denote male family members, circles female family members, solid symbols affected family members, and symbols with a slash deceased family members. Plus signs denote markers typed in the genome-wide screening. The order of markers and genetic distances indicated were derived from data from Genethon or the Cooperative Human Linkage Center.[16,20] The numbering of subjects corresponds to the pedigree in Newport et al.[12] Vertical lines represent the region of homology identified on affected chromosomes. Positions of meiotic recombination are indicated by dashes. The asterisk denotes a single allele in this region for which Subject III-6 was not homozygous. Subject II-3 was not typed for marker D6S1675 (ND).

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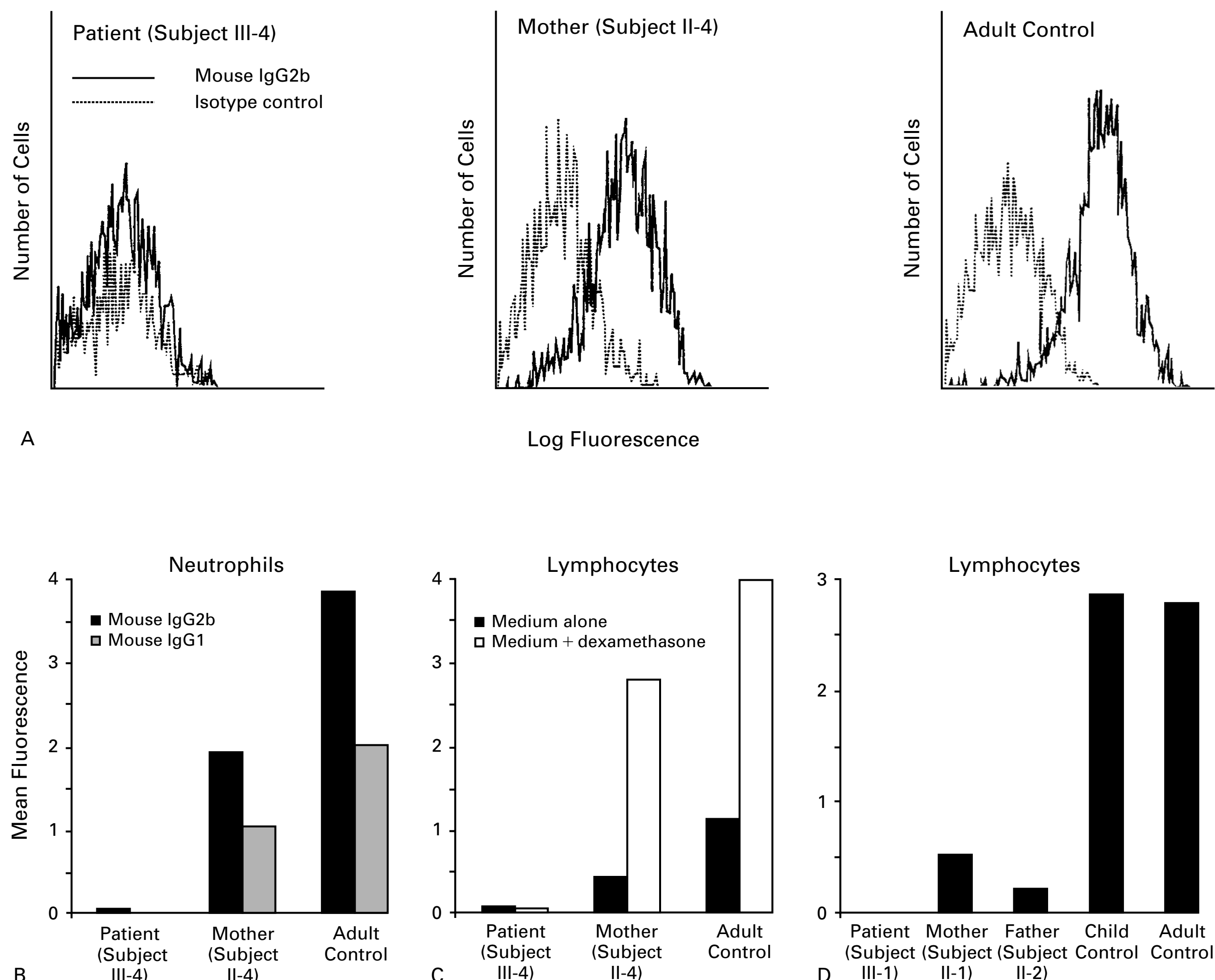

Figure 2. Level of Expression of Interferon-γ Receptor 1 on the Surface of Peripheral-Blood Cells from the Patients, Their Parents, and Healthy Controls.

There was little or no expression of interferon-γ receptor 1 on the surface of the patients' cells and an intermediate level of expression on the parents' cells. Panel A shows the binding of monoclonal antibody against interferon-γ receptor 1 (mouse IgG2b) to fresh monocytes from Subject III-4, his mother (Subject II-4), and a healthy adult control. The solid line indicates binding of the specific antibody; the dotted line indicates binding of an appropriate isotype control antibody. No increase in fluorescence above background levels was detected on the patient's cells (mean fluorescence, 2.42; background, 2.34). There is clear binding to adult control cells (mean fluorescence, 8.53; background, 2.54) and an intermediate level of binding to the maternal cells (mean fluorescence, 5.61; background, 2.13).

Panel B shows the binding of two monoclonal antibodies against interferon-γ receptor 1 to fresh neutrophils from a patient (Subject III-4), his mother (Subject II-4), and a healthy adult control. Panel C shows the binding of monoclonal antibody against interferon-γ receptor 1 (mouse IgG1) to lymphocytes from a patient (Subject III-4), his mother (Subject II-4), and a healthy adult control. Solid bars represent binding to cells cultured in medium alone, and open bars represent binding to cells incubated overnight with dexamethasone. Panel D shows the binding of monoclonal antibody against interferon-γ receptor 1 (mouse IgG2b) to stored lymphocytes from a patient (Subject III-1), his parents (Subjects II-1 and II-2), and healthy adult and age-matched child controls. In each case values have been adjusted for the level of background fluorescence.

Northern analysis of mRNA from EBV-transformed B lymphocytes showed no difference in the expression of the gene for interferon-γ receptor 1 between affected children and an unrelated control (data not shown). The cDNA was prepared from the EBV-transformed lymphocytes by reverse-transcription PCR and sequenced. A substitution of A for C, which results in a stop codon, was identified at position 395 of the coding sequence in all affected children (Fig. 3A).

In order to validate this finding in genomic DNA, a PCR assay was designed that incorporates the site of the mutation into a *Tsp*45I restriction site (as described in the Methods section). The affected chil-

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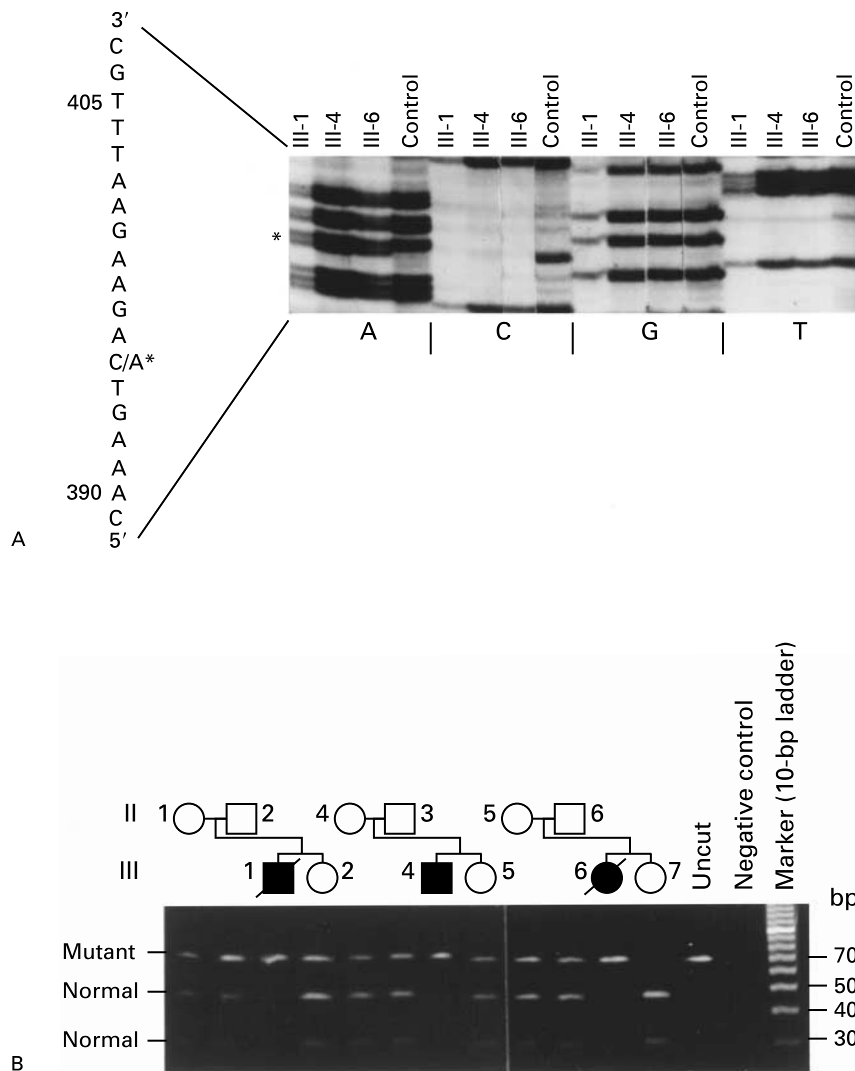

Figure 3. Characterization of the Mutation in the Gene for Interferon-γ Receptor 1 in Patients with Disseminated Mycobacterial Infection and Their Families.

Panel A shows the sequence of the cDNA for the gene for interferon-γ receptor 1 in three of the affected children (Subjects III-1, III-4, and III-6) and an unrelated, unaffected control. The asterisk indicates the substitution of A for C at position 395. The A tracks from each subject were loaded next to each other, as were the C, G, and T tracks. Panel B shows the results of the PCR assay that used genomic DNA. The symbols are defined in Figure 1. The affected children (Subjects III-1, III-4, and III-6) all have a single uncut band and are homozygous for the mutation. Their parents have both cut and uncut fragments and are all heterozygous for the mutation. Subject III-7 is homozygous for the normal sequence.

dren are homozygous for the mutation, whereas their parents are heterozygous carriers (Fig. 3B). Siblings III-2 and III-5 are heterozygous, whereas sibling III-7 is homozygous for the normal sequence (Fig. 3B), as they are for the microsatellites around the mutation (Fig. 1).

Consistent with the mutation in the gene for interferon-γ receptor 1 and the absence of interferon-γ–receptor 1 protein on cell surfaces as detected by the monoclonal antibodies, TNF-α production in response to interferon-γ was markedly lower in the surviving affected child than in healthy controls; his parents had intermediate responses (Fig. 4).

DISCUSSION

We have shown that a novel immunodeficiency predisposing affected persons to mycobacterial infection is caused by a mutation in the gene for interfer-

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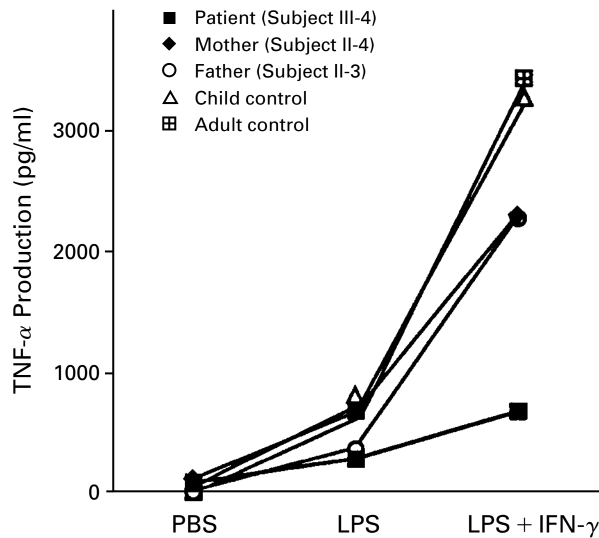

Figure 4. TNF-α Production in Response to Stimulation of Whole Blood with Phosphate-Buffered Saline (PBS), Lipopolysaccharide (LPS) Alone, or Lipopolysaccharide and Interferon-γ (IFN-γ) in the Surviving Affected Child, His Parents, and Control Subjects.

The affected child (Subject III-4) has markedly reduced TNF-α production in contrast to a control child, who has localized atypical mycobacterial infection, and a healthy adult control, whereas his parents have intermediate responses.

on-γ receptor 1. We have mapped the defect to the same chromosomal region as the receptor gene and identified a point mutation in the coding region. The substitution of A for C at position 395 results in a new stop codon and in the production of a truncated protein that would be predicted to lack the membrane-binding region and the intracellular domain and would be unlikely to be expressed on the cell surface (Fig. 5A).

The major defects in immunologic function observed in the affected patients are the failure of interferon-γ to up-regulate the production of TNF-α by macrophages and defective antigen processing and presentation.[11,24] Unlike the situation in cells from healthy controls, in monocytes from the patients exogenous interferon-γ induces only a small increase in TNF-α production in vitro (Fig. 4). These findings are explained by our molecular studies showing a mutation in the gene for interferon-γ receptor 1 and by our cellular studies confirming the absence of the expression of interferon-γ receptor 1 on cell surfaces as detected by the binding of monoclonal antibodies to the extracellular domain of the receptor. However, given the absence of interferon-γ receptor 1 on cell surfaces, it is surprising that we observed any up-regulation of monocytes by interferon-γ. It is also surprising that there appeared to have been clinical improvement associated with the administration of interferon-γ to three of the patients.[11] This paradox may be explained by considering the function of the various components of the interferon-γ receptor (Fig. 5B).

Interferon-γ induces cellular activation by binding to a receptor complex consisting of at least two subunits: the interferon-γ–binding subunit (interferon-γ receptor 1) and a chromosome 21–encoded transmembrane accessory factor (interferon-γ receptor 2) (Fig. 5B).[25] Both components of the receptor are thought to be required for normal signal transduction. Binding of interferon-γ induces dimerization of the interferon-γ receptor 1, which associates with interferon-γ receptor 2 (Fig. 5B). There is evidence that interferon-γ interacts with both interferon-γ receptor 1 and interferon-γ receptor 2 during the process of association of the two receptor proteins.[23] The Janus protein kinases Jak 1 and Jak 2 are associated with the intracellular domains of interferon-γ receptor 1 and interferon-γ receptor 2, respectively, and are brought together and activated by phosphorylation by the binding of interferon-γ to the receptor complex. This results in the phosphorylation of tyrosine at position 457 of the interferon-γ receptor 1 chain and produces a binding site for Stat 1α (signal transduction and activation of transcription protein), leading to the phosphorylation, homodimerization, and subsequent dissociation of Stat 1α from the interferon-γ–receptor complex.[26] The Stat 1α dimer translocates to the nucleus and interacts with γ-activation sequences in the promoter regions of interferon-γ–inducible genes, resulting in their transcription (Fig. 5B).

The mutation present in our patients severely disrupts the function of the interferon-receptor complex, since interferon-γ receptor 1 is required both for binding of interferon-γ and for signal transduction. The small residual response to interferon-γ observed in our patients and the apparent clinical response to exogenous interferon-γ have a number of possible explanations. The truncated extracellular domain of interferon-γ receptor 1 may be transported to the cell surface and shed into the plasma, since the membrane anchor is absent. Interferon-γ may associate with the truncated receptor in the plasma, and the complex may interact with interferon-γ receptor 2, allowing some signal transduction through this receptor (Fig. 5B). Alternatively, interferon-γ may bind directly to interferon-γ receptor 2 and induce a minor degree of cellular activation. In the absence of the transmembrane region of interferon-γ receptor 1, signaling through the Janus kinases is likely to be markedly reduced. Finally, interferon-γ may induce a milder degree of cellular activation through an as yet unidentified additional receptor. Most of what is known about the functions of the human interferon-γ–receptor complex has been learned by studying human–rodent chimeras in which individual components of the receptor complex can be knocked out.[23] Our patients

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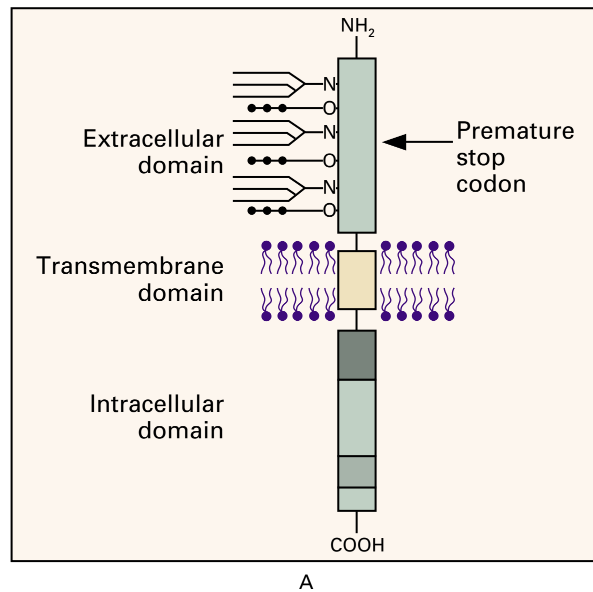
Figure 5. Structure of the Interferon-γ–Receptor 1 Protein and a Model of the Interferon-γ Receptor Complex.

Panel A shows the structure of the interferon-γ–receptor 1 protein and the position of the mutation. The structure was based on the model of Farrar and Schreiber.[22] The mutation results in a premature stop codon. Interferon-γ–receptor 1 is a transmembrane protein with an extracellular domain of 228 amino acids, a transmembrane domain of 23 amino acids, and an intracellular domain of 221 amino acids that is rich in serine and threonine residues, which are phosphorylated on ligand binding. The shaded sections represent functionally important areas required for signal transduction. The mutant protein lacks the transmembrane and intracellular domains required for signal transduction. Panel B shows a model of the interferon-γ–receptor complex and signal transduction based on the data of Kotenko et al.[23] On the left side both interferon-γ receptor 1 (green bars) and interferon-γ receptor 2 (red bars) extend through the cell membrane. The protein kinases Jak 1 and Jak 2 are in close association with the two chains of the receptor. In the middle of the panel, on binding of interferon-γ (IFN-γ; yellow boxes), the chains associate, triggering phosphorylation (●), which exposes a binding site for Stat 1α. The right side of the panel shows a proposed model to explain residual cell activation in affected patients. Interferon-γ may associate either with the truncated interferon-γ receptor 1 or with interferon-γ receptor 2 directly.



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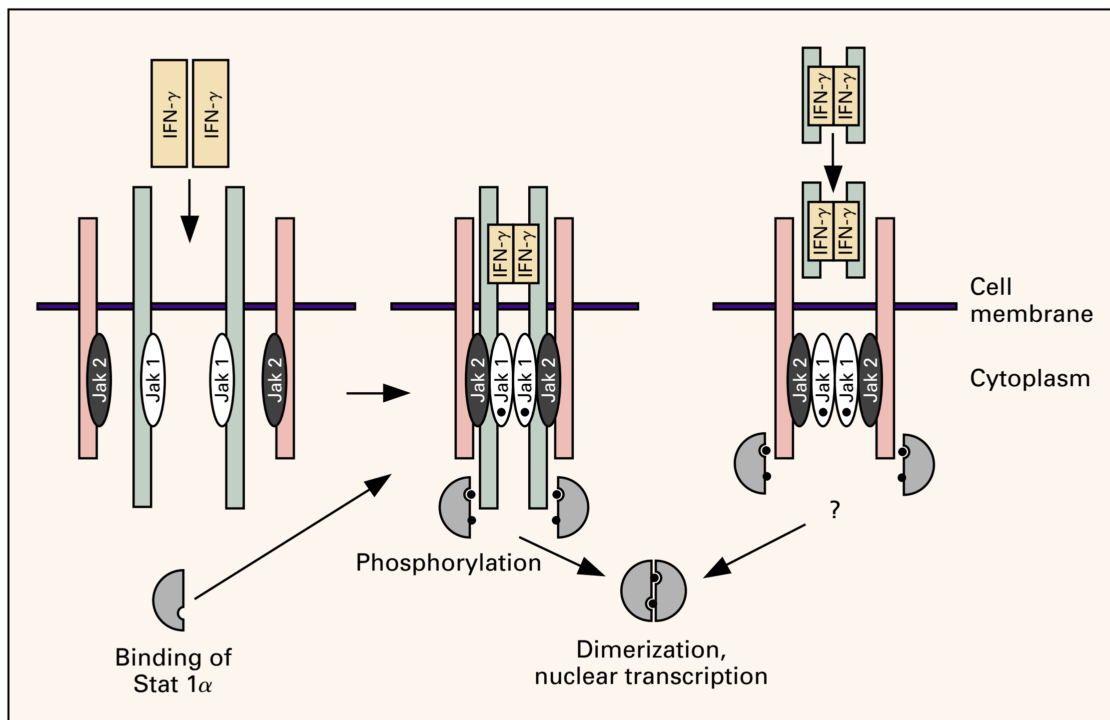

B

provide an opportunity to study the receptor in what might be considered a knockout in humans, and further studies are required to explain their residual response to interferon-γ fully.

A large literature documents the importance of interferon-γ in the up-regulation of murine macrophage function to control intracellular organisms, including mycobacteria, salmonella, and leishmania.[27] Interferon-γ produced by T cells and natural killer cells induces macrophage activation, resulting in increased production of interleukin-1 and TNF-α,[28] enhanced antigen presentation,[29] and increased production of nitric oxide[30] and reactive-oxygen intermediates.[31] Murine macrophages activated by inter-

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feron-γ are able to limit the growth of mycobacteria in vitro, and there appears to be synergy between interferon-γ and TNF-α.[32] Mice in which the gene for interferon-γ has been disrupted have defective production of macrophage antimicrobial products and reduced expression of major-histocompatibility-complex class II antigens and die of disseminated mycobacterial infection.[10] Moreover, mice lacking interferon-γ receptor 1 have a similar phenotype to those with disruption of the interferon-γ gene and are susceptible to infection with intracellular pathogens.[7] Despite these data in mice, there is no clear evidence that interferon-γ enhances the killing of mycobacteria in vitro by human macrophages.[33-35] However, treatment with interferon-γ resulted in clinical improvement in a group of patients with refractory disseminated nontuberculous mycobacterial infection.[11,36]

A striking feature of the immune defect in these children is that it is highly specific for mycobacteria and possibly other intracellular pathogens such as salmonella. They have not had infections with conventional bacteria or fungi and have coped with viral infections normally. The interferon-γ pathway has been implicated in the immune response to virtually all infectious agents, including fungi, parasites, and bacteria.[37] The specificity of the defect in these children suggests that there must be a redundancy of immunologic mechanisms against most of these pathogens but not against mycobacteria. Perhaps the success of mycobacteria as human pathogens is due to the absence of overlapping immunologic responses able to control their growth in humans.

Elucidation of the basis of this rare immunodeficiency not only provides important information on the role of the interferon-γ pathway in human immunity, but also identifies a gene with a potentially crucial role in determining susceptibility to mycobacterial disease. Clearly, the complete absence of interferon-γ receptor 1 on cell surfaces induces so profound an immune defect that such mutations are unlikely to explain mycobacterial susceptibility in the general population. However, the existence of heterozygous carriers of the defect or more subtle variations in this gene might explain differences in mycobacterial susceptibility within the population. The interferon-γ pathway may be an important potential target for immunotherapy of mycobacterial infections.

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