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Human Complete Stat-1 Deficiency Is Associated with Defective Type I and II IFN Responses In Vitro but Immunity to Some Low Virulence Viruses In Vivo

Ariane Chappier,* Robert F. Wynn, † Emmanuelle Jouanguy,* Orchidée Filipe-Santos,* Shenying Zhang,* Jacqueline Feinberg,* Kay Hawkins, † Jean-Laurent Casanova,* and Peter D. Arkwright 2‡

The autosomal recessive form of human complete Stat-1 deficiency is a rare disorder, thus far reported in two unrelated patients, both of whom developed disseminated bacillus Calmette-Guérin (BCG) and subsequently died of viral illnesses before detailed studies of the condition could be performed. We describe a third patient with complete Stat-1 deficiency and disseminated BCG infection, who died 3 mo after bone marrow transplantation. The patient’s EBV-transformed B cells did not express Stat-1 protein and did not activate Stat-1-containing transcription factors. We also report the ex vivo responses of a Stat-1-deficient patient’s fresh blood cells to IFN-γ and the in vitro responses of a SV40-transformed fibroblastic cell line to IFN-γ and IFN-αβ. There was no response to IFN-γ in terms of IL-12 production and HLA class II induction, accounting for vulnerability to BCG. Moreover, IFN-αβ did not suppress HSV and vesicular stomatitis virus replication in fibroblasts, although in vivo the patient was able to successfully clear at least some viruses. This study broadens our understanding of complete Stat-1 deficiency, a severe form of innate immunodeficiency. Stat-1 deficiency should be suspected in children with severe infections, notably but not exclusively patients with mycobacterial or viral diseases. The Journal of Immunology, 2006, 176: 5078–5083.

Characterization of signaling pathways through which IFNs induce gene expression led to the discovery of a family of transcription factors that directly link cell surface receptors to nuclear events (1). Stat-1 is the major intracellular mediator of IFN-γ effects on gene transcription and also for IFN-αβ. It is activated by tyrosine phosphorylation when IFNs bind to their receptors (2). Activation results in the formation of homo- or heterodimers, which translocate to the nucleus where they act as IFN-responsive gene transcription factors by binding to discrete cis-acting regulatory sequences in DNA (3). Stat-1 homodimers (γ-activating factor (GAF)3) are involved in the response to IFN-γ, whereas Stat-1/Stat-2/IFN regulatory factor 9 heterotrimers (IFN-stimulated gene factor 3 (ISGF3)) act largely as response elements for type I IFNs.

The ability of human macrophages to respond to IFN-γ is critical for the development of immunity and thus eradication of mycobacteria (4). Inherited disorders resulting in either defective production of IFN-γ or response of the macrophages to IFN-γ are associated with disseminated mycobacterial infections. IFN hyporesponsiveness or nonresponsiveness has been reported in patients with both heterozygous and homozygous STAT1 mutations. Heterozygous STAT1 mutations have been described in two patients who were susceptible to mycobacterial but not viral infections (5). The first case was a 33-year-old French woman who developed disseminated BCG infection in childhood from which she recovered. She remains well at last follow-up at age 37, as are her affected 7- and 4-year-old children not reported in Dupuis’s study. The second case is a 10-year-old American girl who developed Mycobacterium avium infection at 6 years old and also remains well at last follow-up at age 14 (J. Harris, unpublished observations).

The clinical features of complete Stat-1 deficiency have only briefly been described previously since diagnoses were made after death. Two unrelated infants from healthy Saudi Arabian parents were found to have homozygous mutations in STAT1 (1758_1759delAG and L600P) (6). Both of these infants developed disseminated BCG infection from which they recovered. One then contracted herpes simplex encephalitis and died, and the other a viral-like illness and died before more detailed investigations could be performed. In the children with complete Stat-1 deficiency, both GAF and ISGF3 were absent. Our present study is the first to describe the complex pathophysiology of the human form of complete Stat-1 deficiency in a third unrelated affected child from Pakistan. It clearly illustrates that Stat-1 is critical for both IFN-γ and IFN-αβ signaling in human cells. In vitro experiments show that without Stat-1, IFNs cannot induce intracellular GAF or ISGF3 activation, cell surface HLA class II expression, or a protective cellular response to at least two DNA and RNA viruses. As well as an inability of cells to respond to the IFN, we show for the first...
time that production of IL-12, IFN-γ, and TNF-α are also defective in this condition.

Materials and Methods

Study subject

Clinical details of the patient and their family are described in Results. Written consent was obtained from the patient’s parents for the laboratory investigations described below.

Whole-blood assays of the IL-12-IFN-γ circuit

Whole-blood assays were performed as previously described (7). Heparinized blood from patients was stimulated in vitro with live BCG (Mycobacterium bovis BCG; Pasteur) at 20 BCG/leukocytes, with BCG plus 5000 IU/ml IFN-γ (Boehringer Ingelheim) and with BCG plus 20 ng/ml rIL-12p70 (R&D Systems). Supernatants were collected after 18 and 72 h of stimulation, and ELISA was performed with specific Abs directed against IFN-γ, IL-12p40, IL-12p70, or TNF-α using the human Quantikine IL-12p70 HS and IL-12p40 kits (R&D Systems) and the human Pelipair IFN-γ and TNF-α kits (Sanquin) according to the manufacturers’ guidelines.

Cell culture and stimulation, DNA and RNA extraction, PCR, sequencing

EBV-transformed B cells (B-EBV) were cultured as previously described (6). Stimulations were performed with 10⁵ IU/ml IFN-γ (Boehringer Ingelheim) and 10⁵ IU/ml IFN-α2b (Schering-Plough). Genomic DNA and total RNA from cell lines were extracted as previously described (6). Amplification and sequencing of genomic DNA and cDNA were done as previously described (6). Primers and PCR conditions are available upon request.

Viral assays

Viral assays were performed as previously described (6). Briefly, skin-derived SV40-transformed fibroblasts were left untreated or were treated with 10⁵ IU/ml IFN-α for 24 h. They were then infected by incubation with various titers of HSV (HSV-1, strain KOS-1, gift from P. Lebon, Saint Vincent de Paul, Paris, France) or vesicular stomatitis virus (VSV, strain Indiana, gift from S. Pellegrini, Pasteur Institute, Paris, France) for 48 h. Viral titers were then determined by visualizing the lysed well.

Western blotting

Whole-cell protein extracts were prepared as previously described and western blotting was performed as previously described (6). The following Abs were used: anti-Stat-1 (BD Transduction Laboratories) and anti-Stat-3 (Santa Cruz Biotechnology).

EMSA

EMSA was conducted as previously described (6). Briefly, cells were stimulated by incubation for 30 min with 10⁵ IU/ml IFN-γ or IFN-α. We incubated 10 μg of nuclear extract with [α-32P]dATP GAS (from FCGRI promoter) or ISRE (from GMP2 promoter).

Flow cytometry (FACS analysis)

Flow cytometry assays were performed as previously described (8, 9). Briefly, skin-derived SV40-transformed fibroblasts were left untreated or were treated with 10⁵ IU/ml IFN-γ for 48 h and were then incubated with PE-anti-HLA-DR Ab (BD Biosciences) for 45 min on ice. PHA-T cell blasts were incubated with IL-12Rβ1- or IFN-γR1-specific Ab (BD Biosciences). Signals were analyzed with a FACSscan and CellQuest software (BD Biosciences).

Fluorescent IL-12 binding

IL-12p70 fluorescence-binding experiments were performed as previously described (9). Briefly, PHA-T cell blasts were incubated with or without 50 ng of IL-12p70 (R&D Systems) for 30 min at 4°C and then with mouse anti-IL-12p40-p70 IgG1, biotinylated rat anti-mouse IgG1, and finally with streptavidin-PE (BD Pharmingen).

Results

Clinical features of complete Stat-1 deficiency

A third child of first-cousin Pakistani parents presented at 3 mo of age with disseminated BCG infection 8 wk after having been given an intradermal BCG vaccine in his left deltoid. The family pedigree is shown in Fig. 1. This patient had high swinging fevers, a generalized maculopapular rash, massive hepatosplenomegaly, and increasing respiratory distress requiring ventilation and intensive care. Acid-fast bacilli were found on bronchoalveolar lavage and lymph node skin and liver biopsies, in which a noncaseating, granulomatous reaction was seen (Fig. 2). The vaccine strain BCG was identified from blood culture isolates and bone marrow, which showed a marked myeloid reaction. The child was started on a combination of isoniazid, rifampicin, ethambutol, and ciprofloxacin at 4.5 mo old. Assessment of the lymphocyte subsets and Ig levels did not suggest an underlying severe combined immunodeficiency/Omenn syndrome.

The patient’s condition transiently improved, but within 2 wk of starting treatment, including the addition of s.c. IFN-γ injections, an intradermal BCG vaccine in his left deltoid. The family pedigree is shown in Fig. 1. This patient had high swinging fevers, a generalized maculopapular rash, massive hepatosplenomegaly, and increasing respiratory distress requiring ventilation and intensive care. Acid-fast bacilli were found on bronchoalveolar lavage and lymph node skin and liver biopsies, in which a noncaseating, granulomatous reaction was seen (Fig. 2). The vaccine strain BCG was identified from blood culture isolates and bone marrow, which showed a marked myeloid reaction. The child was started on a combination of isoniazid, rifampicin, ethambutol, and ciprofloxacin at 4.5 mo old. Assessment of the lymphocyte subsets and Ig levels did not suggest an underlying severe combined immunodeficiency/Omenn syndrome.
A severe inflammatory hepatitis with intrahepatic biliary obstruction developed. Liver biopsy showed an extensive inflammatory infiltrate blocking portal ducts, but no acid-fast bacilli or hepatic necrosis to suggest rampant BCG infection, or drug-induced liver damage. Hepatitis virus serology was negative. The jaundice responded to high-dose i.v. methylprednisolone at an initial dose of 20 mg/kg per day with gradual tapering in combination with tacrolimus and infliximab infusions, although the liver remained grossly enlarged and the patient continued to require assisted ventilation.

Although the molecular diagnosis had not yet been confirmed, a matched sibling bone marrow transplant was performed at 8 mo of age, with preconditioning using alemtuzumab, fludarabine, and melphalan. Prednisolone and tacrolimus were continued throughout as prophylaxis against graft-vs-host disease. The patient engrafted slowly (CD3+/H11001 count at 2.5 mo of 0.3/H1100310⁹/L) and continued to require ventilation support, regular platelet transfusions, and had persisting massive hepatosplenomegaly. At 70 days post-transplantation, the patient developed a fulminant EBV infection with increasing respiratory failure. Although the EBV was cleared from the patient’s blood and respiratory secretions using rituximab and donor T cell infusions, multiorgan failure developed and this patient died at 11 mo of age, 91 days posttransplant.

Apart from the infection with EBV, a number of viruses were isolated from the child during his short life, but all cleared spontaneously.

**FIGURE 3.** Cytokine production in the supernatant of whole blood from the patient (■) and a healthy control (○), unstimulated (medium) or stimulated for 72 h with live BCG alone or BCG + IL-12 or IFN-γ. The levels of IL-12p40, IL-12p70, TNF-α, and IFN-γ in the supernatant were determined by ELISA.

**Table I.** Viral isolation and serology during lifetime of the patient

<table>
<thead>
<tr>
<th>Age of patient</th>
<th>Virus Detection (PCR, IF) or Isolation by Culture</th>
<th>Viral Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mo (May 16, 2004)</td>
<td>Polio III by PCR (NPA) but not by culture of NPA or stools</td>
<td>Polio I &lt;10 titer, II 80 titer, III &gt;160 titer</td>
</tr>
<tr>
<td>4 mo (May 15, 2004)</td>
<td>Parainfluenza II by PCR (NPA) but not by IF or culture Polio III cleared</td>
<td>Hepatitis B surface Ag, anti-hepatitis C virus core Ag, anti-hepatitis C, HIV, HSV I/II, CMV, VZV: all negative</td>
</tr>
<tr>
<td>5 mo (August 16, 2004)</td>
<td>No viruses by PCR, IF, or culture (NPA, BAL, and stools)</td>
<td>On IVIG: serology not possible</td>
</tr>
<tr>
<td>8 mo (November 6, 2004)</td>
<td>No viruses by PCR, IF, or culture (NPA, BAL, blood)</td>
<td></td>
</tr>
<tr>
<td>10 mo (January 14, 2005)</td>
<td>Rhinovirus isolated (NPA) acute EBV PCR peaked at 538,000 copies/ml (blood)</td>
<td></td>
</tr>
<tr>
<td>11 mo (February 10, 2005)</td>
<td>Rhinovirus and EBV negative (NPA and blood)</td>
<td></td>
</tr>
</tbody>
</table>

* NPA, Nasopharyngeal aspirate; IF, immunofluorescence; BAL, bronchioalveolar lavage; VZV, varicella zoster virus.
without leading to life-threatening disease or requiring the use of antiviral agents (Table I). At 2 mo old, the child developed an intercurrent upper respiratory tract infection associated with corneal symptoms, which settled spontaneously within a week. The child had recently had the first scheduled primary vaccinations, including the live oral polio vaccine. Polio virus type III was isolated by PCR from a nasopharyngeal aspirate but was not cultured either from this aspirate or from stools. Serology at 5 mo confirmed that the child had made a good response to this virus with a polio type III IgG of >160 titer (polio I, 10 titer and polio II, 80 titer). Paramyxovirus type II was identified from the bronchoalveolar lavage by PCR at 3 mo of age, but was negative on repeated immunofluorescence and viral culture. Serological testing for this virus was not available. Posttransplantation, rhinovirus was isolated from nasopharyngeal aspirates but cleared spontaneously over succeeding few weeks.

**Complete Stat-1 deficiency**

The clinical features of severe disseminated BCG infection were in keeping with a defect in the IFN-γ pathway and thus further in vitro investigations were organized. There was a complete inability of BCG to stimulate the patient’s blood leukocytes to produce either IL-12 (p40 and p70) or IFN-γ over and above background (Fig. 3). Production of BCG-induced TNF-α was also markedly suppressed. IL-12, IL-12R, IFN-γ, and IFN-γ receptor were all sequenced but no mutation was found. Flow cytometric analysis showed normal IFN-γR1 and IL-12Rβ1 cell expression and normal binding of IL-12p70 on the patient’s PHA blasts (Fig. 4). Sequencing of the STAT1 gene revealed a homozygous 1928insA mutation (Fig. 5A). This mutation was associated with a frame shift and led to a stop codon at position 1936–1938 in STAT1 cDNA. Examining the expression of Stat-1 and Stat-3 by Western blot, B-EBV cells of the patient showed no expression of Stat-1, but normal expression of Stat-3 (Fig. 5B).

**Nonresponsiveness to IFNs in vitro**

The role of Stat-1 in IFN signaling was studied using a number of assays. First, intracellular signaling was assessed in the patient’s Stat-1 deficient cells and a control after stimulation with IFN-γ or IFN-α by measuring binding of GAF and ISGF3 on γ-activating sequences (GAS) and IFN-stimulated response element (ISRE)-radioactive probes, respectively (Fig. 5, C and D). There was a complete lack of GAS- and ISRE-binding protein expression after IFN-γ and IFN-α stimulation. Second, induction of HLA-DR expression on the cell surface of SV40-transformed fibroblasts by IFN-γ was investigated. IFN-γ was unable to up-regulate HLA-II expression in the patient’s Stat-1-deficient cells (Fig. 6). Third, the functional effects of this defect were studied by measuring the ability of IFN-α to suppress HSV and VSV replication. Unlike the control where IFN-α reduced replication of both HSV and VSV, IFN-α had no effect on the levels of these viruses in the patient’s Stat-1-deficient fibroblasts (Fig. 7).

**Discussion**

This is the first study to describe the complex ex vivo immune consequences of the human form of complete Stat-1 deficiency, as the definitive diagnosis has only previously been made after death. We show that complete Stat-1 deficiency in humans is associated with substantially lower ex vivo IL-12, IFN-γ, and also TNF-α production in response to mycobacteria (BCG) without augmentation by exogenous IFN-γ. Our observations are in keeping with recent studies in mice which have demonstrated that Stat-1 augments the production of both IL-12 and TNF-α (10, 11). Our findings highlight the fact that IFN-γ levels usually used to screen for IFN pathway defects are low in complete Stat-1 deficiency, in contrast to the raised levels of these cytokines observed in IFN resistance due to complete IFN-γ receptor deficiencies (12).

We confirm and extend our previous observations of complete Stat-1 deficiency in terms of defective GAF and ISGF3 expression (6) in showing that this disease is also associated with the inability to up-regulate class II MHC expression on the surface of SV40-transformed fibroblasts. The complete functional resistance to the action of IFN-γ and the secondary effect of attenuated TNF-α production, as detailed above, would explain the heightened predisposition of patients with complete Stat-1 deficiency to disseminated infection with BCG and other opportunistic mycobacteria (4, 13).

We also demonstrate the functional impact of the human form of complete Stat-1 deficiency on immunity to viruses. Our in vitro experiments show that IFN-α is unable to prevent replication of a RNA and a DNA virus in Stat-1-deficient human fibroblasts. This observation would explain the death of a previous complete Stat-1-deficient patient from fatal herpes simplex encephalitis (6). It is also in keeping with evidence from a homozygous STAT1 knockout mice model which has a susceptibility to a number of viruses including HSV, VSV, and mouse hepatitis virus (14, 15). However, our careful in vivo observations show that immunity to some viruses remains sufficient to protect the patient against severe infection. Our patient was able to mount a protective immune response and clear the attenuated polio type III virus in the Sabin vaccine given at 2 mo of age. We were able to detect the virus by
PCR from nasopharyngeal secretions and demonstrate that the child mounted an excellent Ab response without developing any neurological sequelae. This may be because of the very low virulence of this virus which rarely causes disease, even in patients with severe combined immunodeficiency (16).

The patient was also able to successfully clear a rhinovirus infection acquired after bone marrow transplantation and a parainfluenza type II infection, confirmed by PCR at 4 mo of age without the development of severe disease. It is not possible to make any conclusions about the posttransplant EBV infection, which could just as easily be explained by the slow engraftment and immunosuppression as to the underlying immunodeficiency. One explanation for these clinical findings is that Stat-1-independent pathways might provide adequate protection against less virulent viruses. In support of this possibility is that Stat-1-independent as well as Stat-1-dependent pathways have recently been shown to control primary dengue virus infections in mice (17, 18).

In addition to the novel findings detailed above, this is the first report of stem cell transplantation being attempted for complete Stat-1 deficiency. The conditioning used in this patient did not reverse the marked hepatic inflammation that was a major clinical
problem pretransplantation and remained an ongoing problem posttransplantation. Inability of standard conditioning regimens to eradicate Stat1-deficient tissue macrophages may be an explanation for the persistent inflammatory reaction, and thus modification of standard protocols may have to be developed, particularly in the more complex cases, if stem cell transplantation is to be successful.

As mentioned above, a severe life-threatening inflammatory hepatitis was a major clinical complication in our patient with Stat1 deficiency and necessitated very large doses of systemic corticosteroids to bring the problem under some control. Stat1-deficient mice can develop life-threatening inflammatory responses due to defective CD4⁺CD25bright regulatory T cells (19). It is possible that human Stat1 deficiency is associated with a similar dysfunction of T regulatory cells, but because in our patient the disseminated BCG infection had triggered the activation of large numbers of CD4⁺ cells, further assessment of CD4⁺CD25bright regulatory T cells by flow cytometry was not possible. Severe reactive inflammation should be considered a possible complication in future patients with this rare condition. Further work is required to unravel the pathophysiology of this problem.

In summary, this definitive report refines our knowledge of the clinical and biological effects of human Stat-1 in vitro and in vivo, illustrating the importance of this molecule to both our susceptibility to viral and intracellular bacterial infections. It defines the profile of in vitro IL-12 and IFN-γ responses indicative of this condition. The complexities of stem cell transplantation for this primary immunodeficiency are also highlighted. Finally, since complete Stat-1 deficiency has now been described in patients of primary immunodeficiency are also highlighted. Finally, since complete Stat-1 deficiency has now been described in patients of primary immunodeficiency, this will help in the genetic counseling of families affected by this severe disorder. The cases of two individuals described here are likely to be additional cases of human complete Stat-1 deficiency, although no complete lack of Stat1 in peripheral blood monocytes was detected in one of these patients in vitro. It is possible that human Stat-1 deficiency is associated with a similar dysfunction of T regulatory cells, but because in our patient the disseminated BCG infection had triggered the activation of large numbers of CD4⁺ cells, further assessment of CD4⁺CD25bright regulatory T cells by flow cytometry was not possible. Severe reactive inflammation should be considered a possible complication in future patients with this rare condition. Further work is required to unravel the pathophysiology of this problem.

Acknowledgments
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Disclosures
The authors have no financial conflict of interest.

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