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Research Article **Immunology**

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Acquired predisposition to mycobacterial disease due to autoantibodies to IFN-γ

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Genetic defects in the IFN-γ response pathway cause unique susceptibility to intracellular pathogens, particularly mycobacteria, but are rare and do not explain mycobacterial disease in the majority of affected patients. We postulated that acquired defects in macrophage activation by IFN-γ may cause a similar immunological phenotype and thus explain the occurrence of disseminated intracellular infections in some patients without identifiable immune deficiency. Macrophage activation in response to IFN-γ and IFN-γ production were studied in whole blood and PBMCs of 3 patients with severe, unexplained nontuberculous mycobacterial infection. In all 3 patients, IFN-γ was undetectable following mitogen stimulation of whole blood, but significant quantities were detectable in the supernatants of PBMCs when stimulated in the absence of the patients' own plasma. The patients' plasma inhibited the ability of IFN-γ to increase production of TNF-α by both autologous and normal donor PBMCs, and recovery of exogenous IFN-γ from the patients' plasma was greatly reduced. Using affinity chromatography, surface-enhanced laser desorption/ionization mass spectrometry, and sequencing, we isolated an IFN-γ–neutralizing factor from the patients' plasma and showed it to be an autoantibody against IFN-γ. The purified anti–IFN-γ antibody was shown to be functional first in blocking the upregulation of TNF-α production in response to endotoxin; second in blocking induction of IFN-γ–inducible genes (according to results of high-density cDNA microarrays); and third in inhibiting upregulation of HLA class II expression on PBMCs. Acquired defects in the IFN-γ pathway may explain unusual susceptibility to intracellular pathogens in other patients without underlying, genetically determined immunological defects.

Introduction

Although one-third of the world's population shows tuberculin skin reactivity indicative of previous exposure to *Mycobacterium tuberculosis*, less than 10% of those individuals develop clinical disease (1). The human immunological mechanisms that distinguish the majority of individuals, in whom these organisms are successfully contained, from the small minority who develop progressive mycobacterial diseases are largely unknown. Furthermore, nontuberculous mycobacteria (NTMs) are ubiquitous organisms, yet clinical disease is virtually only seen in patients with underlying immunodeficiencies (2).

In 1996 our group, and that of Casanova, reported that mutations in the gene encoding the IFN-γ receptor, leading to absence of expression of the IFN-γ receptor 1 chain, were the cause of familial susceptibility to mycobacterial infection (3, 4). Several different mutations in both genes encoding the IFN-γ receptor chains have subsequently been identified as the cause of disseminated atypical mycobacterial infections (5–9), disseminated BCG-osis (10–12), or tuberculosis (TB) (13). Individuals with defective IFN-γ receptor expression or function have a widespread defect in macrophage activation, which results in reduced production of TNF-α and other proinflammatory cytokines in response to IFN-γ and endotoxin (2, 3, 7, 9, 14), defective MHC class II expression in response to IFN-γ or antigenic stimulation, and reduced ability to present antigen to T cells (13, 15). A similar susceptibility to mycobacterial infection due to mutations in the genes for the IL-12 p40 subunit and the IL-12 Rβ1 subunit (16–18) and in the *STAT1* gene (19) has also been reported. These observations have established that upregulation of macrophage mycobactericidal mechanisms through the IFN-γ or IL-12 pathway is critical for a successful immune response to mycobacteria (20, 21). However, genetic defects in the IFN-γ pathway are rare and are unlikely to explain the majority of cases in both adults and children with unusual susceptibility to mycobacteria. In this report, we describe 3 patients with severe, progressive NTM infection, in whom autoantibodies against IFN-γ have resulted in a phenotype similar to that seen with mutations in the IFN-γ response pathway.

Results

Case descriptions

Patient 1. A 46-year-old, previously healthy UK resident presented in August 1993 with a 2-week history of weight loss, joint pains, abdominal pain, and fever. She was initially thought to be suffering from Crohn disease and was treated with corticosteroids and

Nonstandard abbreviations used: LTBI, latent TB infection; NTM, nontuberculous mycobacterium; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; SELDI, surface-enhanced laser desorption/ionization; TB, tuberculosis.

Conflict of interest: The authors have declared that no conflict of interest exists.

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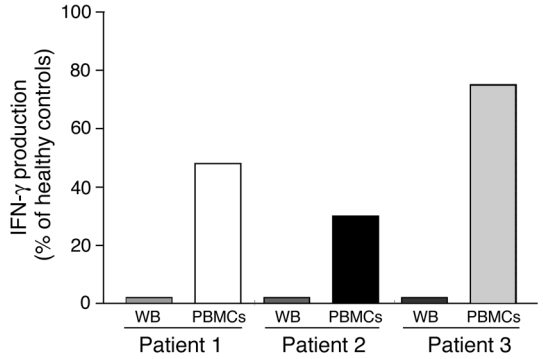

Figure 1
IFN-γ production in response to mitogen stimulation in whole blood (WB) and isolated PBMCs. IFN-γ was not detected in patients' plasma derived from whole blood after stimulation with PHA (or PMA/ionomycin in patient 2), whereas large quantities of IFN-γ were detected when isolated PBMCs were studied in the absence of patient plasma. IFN-γ production in PBMCs is expressed as a percentage of that in blood of healthy controls studied in the same experiments. Supernatants from triplicate samples of whole blood or PBMCs were pooled for each patient and control and assayed in duplicate by ELISA. The coefficients of variation (CVs) in each assay were less than 5%.

an elemental diet. Her symptoms worsened despite this treatment, and she subsequently underwent a right hemicolectomy, after which her condition improved. However, over the next year, she again developed abdominal and bone pains, fevers, and weight loss, followed by stridor and multiple soft tissue masses over her left clavicle and neck. Tracheal obstruction due to a granulomatous mass was demonstrated, as well as evidence of multiple inflammatory bone lesions. A biopsy of the neck masses and clavicular mass showed multiple granulomata containing acid-fast bacilli. *Mycobacterium avium intracellulare* (MAI) was subsequently cultured from the biopsies of the neck mass and bones. She was treated with rifabutin, clarithromycin, ethambutol, and clofazimine but continued to deteriorate, exhibiting wasting and fevers. Treatment with IFN-γ (Immukin; Boehringer Ingelheim) was commenced at a slowly escalating dose. She experienced severe malaise, myalgia, and fevers with the initial doses but subsequently tolerated it well. She progressively improved on this treatment, gained weight, and became symptom free. All anti-microbials were discontinued after 42 months. She currently remains healthy on treatment with 100 μg/m^2 IFN-γ 3 times weekly.

Patient 2. A 32-year-old South African male presented to hospital in November 2000 with back pain and episodic fevers. He had been treated with nonsteroidal antiinflammatory agents, antibiotics, and a 3-month course of corticosteroids without improvement. On admission, he was found to have a paraspinal mass and wedge compression of the T7 vertebra. Fine needle biopsy revealed acid-fast bacilli, and cultures yielded MAI. He was treated with clarithromycin, rifabutin, and ethambutol and slowly improved.

Patient 3. A 59-year-old UK resident presented in April 2002 with a 6-week history of fever, breathlessness, and weight loss. Radiography showed extensive destruction of her left lung, with consolidation, cavitation, and bronchiectatic changes in both lung fields. *Mycobacterium fortuitum* was cultured from her bronchoalveolar fluid, as well as *Aspergillus* species. Despite treatment with nontuberculous mycobacterial drugs and amphotericin, her condition deteriorated until she required ventilatory assistance for respiratory failure. With ongoing treatment over time, she slowly improved.

IFN-γ production in response to mitogen in whole blood and isolated PBMCs

Stimulation of whole blood from healthy controls using the mitogen phytohemagglutinin (PHA; or phorbol 12-myristate 13-acetate/ionomycin [PMA/ionomycin] in the case of patient 2) resulted in the production of large quantities of IFN-γ. In contrast, when the patient's whole blood was stimulated, IFN-γ was not detectable in the supernatant plasma. However, in the absence of their own plasma, the patients' washed PBMCs produced large quantities of IFN-γ in response to mitogens, although less than that of the controls (Figure 1). These findings suggested the presence of a plasma factor inhibiting the detection of IFN-γ.

Impaired upregulation of macrophage TNF-α production in response to exogenous IFN-γ

The ability of purified IFN-γ to activate macrophages to produce TNF-α in response to endotoxin was assessed. In the presence of control serum, production of endotoxin-induced TNF-α increased when PBMCs from either patients or healthy controls were exposed to IFN-γ. In contrast, in the presence of the patients' serum, TNF-α production by either the patients' or control PBMCs was markedly diminished upon exposure to a range of concentrations of exogenous IFN-γ (Figure 2).

Recovery of exogenous IFN-γ from patients' or control sera

In order to establish whether the patients' sera contained a factor that neutralized or degraded IFN-γ, we added increasing concentrations of recombinant IFN-γ to patients' or control serum and then measured the concentration of IFN-γ recovered. The quantity of exogenous IFN-γ detected in patients' serum was markedly reduced. Only upon addition of 10^6 pg/ml was IFN-γ measurable (Figure 3).

Purification and identification of the IFN-γ–neutralizing factor from patients' sera

To purify the IFN-γ–neutralizing factor, we passed patient or control serum through an IFN-γ affinity column and then sequentially eluted the serum factors binding to the IFN-γ with increasing concentrations of sodium chloride followed by 0.1 M glycine HCl. The IFN-γ–neutralizing activity was recovered only in the fractions eluting at low pH. No activity was found in any other fraction or in fractions from control serum subjected to identical fractionation procedures.

To establish the identity of the IFN-γ–neutralizing factor, we subjected the active IFN-γ–neutralizing fractions recovered from the affinity column to analysis by SDS-PAGE electrophoresis, surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, and N-terminal sequencing. SDS-PAGE analysis of the active fractions from patient 1 revealed a single band with a molecular weight of 150 kDa on unreduced gels, whereas the other patients had high-molecular-weight bands above 400 kDa. After treatment with dithiothreitol, the high-molecular-weight bands were reduced to 2 smaller bands of approximately 50 kDa and 30 kDa, respectively (data not shown). The bands were then either electroblotted to membranes for Edman degradation or excised for peptide mapping. The tryptic peptides were resolved using SELDI mass spectrometry. ProFound software (http://

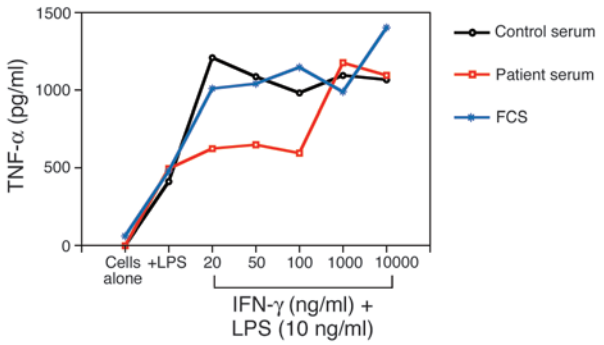

Figure 2
IFN-γ–induced TNF-α upregulation is inhibited in the presence of patients' sera. PBMCs from healthy donors showed marked upregulation of TNF-α production when exposed to concentrations of IFN-γ as low as 20 ng/ml in the presence of control serum (black) or fetal calf serum (blue). Addition of IFN-γ failed to upregulate TNF-α production in the presence of patients' serum (red) at concentrations of IFN-γ from 20 to 100 ng/ml, and upregulation was only seen at very high concentrations. The figure shows a representative experiment using serum from patient 1. All samples were set up in triplicate and supernatants pooled for IFN-γ ELISA measurements in duplicate wells. The CV between duplicates was less than 5%.

prowl.rockefeller.edu/profound_bin/WebProFound.exe) was used to search the NCBI database in order to establish the identities of the proteins and their peptic digests. Finally, confirmation of the identity of the proteins was established by N-terminal sequencing undertaken in the Department of Biochemistry, University of Newcastle (United Kingdom). The IFN-γ–neutralizing factor in patient 1 was thus identified as an IgG_3 antibody, and those of patients 2 and 3 were found to be IgM antibodies.

Specificity of anti–IFN-γ antibody and determination of serum anti–IFN-γ IgG and anti-IgM titers by ELISA

In order to establish whether the identified antibodies were specific to our NTM patients or could also be found in patients with other mycobacterial diseases, such as TB, we repeated complementary experiments using serum from 13 patients with TB, 8 tuberculin-positive healthy contacts of TB patients (patients with latent TB infection [LTBI]), and 11 tuberculin-negative healthy individuals.

In contrast to our findings in the 3 NTM patients, TNF-α production in response to LPS and IFN-γ was not impaired in PBMCs from healthy controls when serum from the 3 groups described above was added. There were no significant differences in the median ratios calculated from the levels of TNF-α produced in response to LPS plus IFN-γ versus LPS alone when plasma from TB patients (median, 615 pg/ml; range, 194–1,011 pg/ml), patients with LTBI (median, 780 pg/ml; range, 708–1,041 pg/ml), and the tuberculin-negative individuals (median, 553 pg/ml; range, 324–776 pg/ml) was used. The amount of exogenous IFN-γ recovered from the serum of the TB patients and patients with LTBI did not differ from that recovered from the serum of tuberculin-negative individuals (data not shown).

Furthermore, we sought evidence that active TB or infection could have produced similar antibodies to those seen in our patients with NTM by using an anti–IFN-γ IgG– or IgM–specific ELISA. No significant ODs were detectable in the TB-infected/diseased individuals (mean, 0.01) compared with the tuberculin-negative individuals (mean, 0.009), and high ODs were detected in the NTM patients' serum (mean, 0.334).

Functional activity of the purified IFN-γ–binding antibody according to high-density cDNA microarrays

In order to confirm that the anti–IFN-γ antibodies were functional, we studied the upregulation of IFN-γ–inducible genes by microarray analysis, in the presence or absence of the purified antibody. As shown in Figure 4, exposure of PBMCs to IFN-γ induced changes in multiple genes. In the presence of the purified anti–IFN-γ antibody, a shift in the gene expression profile was seen, which was not observed in the presence of the nonspecific control antibody. The majority of genes of known function that were identified to be induced (more than 2.5-fold) by IFN-γ treatment at each time point (determined by subtracting the mean $\log_2$ ratio of the abundance of mRNA of untreated from that of IFN-γ–treated PBMCs) were expressed at an at least 2.5-fold lower level after treatment with IFN-γ and the purified anti–IFN-γ antibody (determined by subtracting the mean $\log_2$ ratio of the abundance of mRNA of the nonspecific antibody–treated from that of the anti–IFN-γ antibody–treated PBMCs). Of the 48 genes of known function upregulated after 2 hours treatment with IFN-γ, 43 (89.6%) showed greater then 2.5-fold reduction in expression in the presence of purified anti–IFN-γ antibody. Similarly, at 6 hours, 41 of 46 genes (89.1%) of known function induced by IFN-γ were repressed. At 21 hours, 111 of 119 (93.2%) known genes induced by IFN-γ were repressed by 2.5-fold or more in response to treatment with the purified anti–IFN-γ antibody.

Expression of the gene encoding TNF-α was downregulated over time in the presence of the antibody (Figure 4, cluster I), which confirmed the impaired upregulation seen in PBMCs as shown in Figure 1. Significance analysis of microarrays (SAM) was used to identify genes significantly differentially expressed throughout the time course between the response of PBMCs to treatment with IFN-γ treatment alone and that to treatment with IFN-γ together with the purified anti–IFN-γ antibody. This unpaired 2-class comparison identified 120 cDNA elements, corresponding to 64

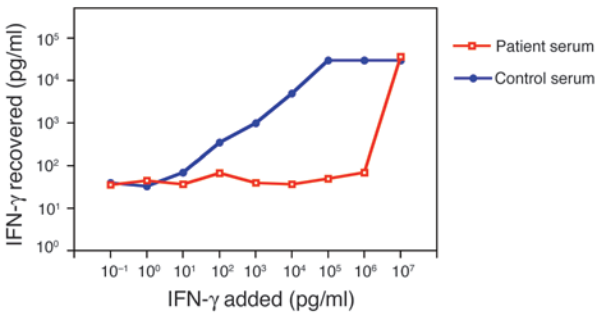

Figure 3
Recovery of IFN-γ from patient or control serum. Increasing concentrations of purified IFN-γ were added to patient (red) or control serum (blue). After 1 hour incubation at room temperature, the concentration of IFN-γ in the serum was determined by ELISA. Exogenous IFN-γ was not recovered from the patients' serum even when added at concentrations 5 logs greater than that required for recovery from normal serum. The figure shows a representative experiment using serum from patient 1. All samples were set up in triplicate and supernatants pooled for IFN-γ ELISA measurements in duplicate wells. The CVs between duplicates were less than 5%.

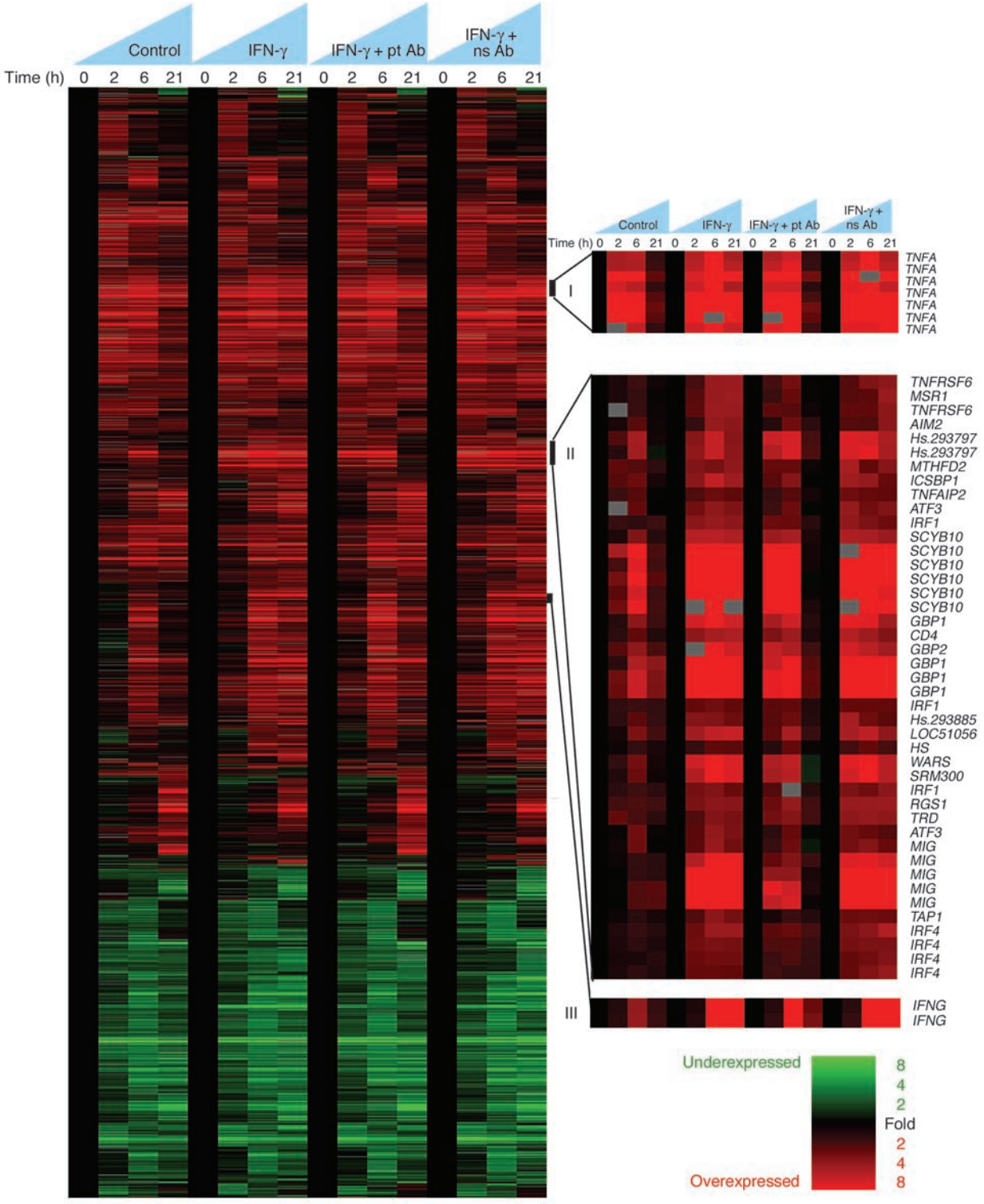

Figure 4

High-density cDNA microarrays showing functional activity of IFN-γ–binding antibody. PBMCs from a healthy donor were exposed to 4 different conditions — no treatment with IFN-γ or antibody (control); IFN-γ alone; IFN-γ with the patient antibody (pt Ab); and IFN-γ with a nonspecific antibody (ns Ab) — each over 4 time points (0, 2, 6, and 21 hours). After data selection and normalization, we filtered for genes demonstrating at least 2.5-fold change from baseline at any 2 of the time points surveyed. The resulting genes were ordered by agglomerative hierarchical clustering (average linkage method) using Cluster software. The genes are displayed in rows, time points in columns. Induced genes are depicted in red, repressed genes in green. Gray represents missing data. When the patient's purified anti–IFN-γ antibody was present with IFN-γ, the gene expression profile most closely resembled that of the control subject. Cluster I shows TNF genes; cluster II, genes most affected by the presence of the antibody; and cluster III, expression of IFN-γ.

unique genes of known function (detailed in Table 1) that were significantly underexpressed on addition of the purified anti–IFN-γ antibody. Many of these genes have been previously shown to be induced by IFN-γ stimulation (22, 23).

In addition, the hypergeometric distribution was used to determine whether the number of genes previously demonstrated to be induced by IFN-γ was significantly enriched in the subset of genes upregulated in response to IFN-γ and downregulated after treatment with the purified anti–IFN-γ antibody. The number of IFN-γ–induced genes in this subset was found to be highly significant ($P = 4.5 \times 10^{-23}$). This microarray analysis demonstrated that the purified anti–IFN-γ antibody recovered from patient 1 was functional and able to repress a subset of genes induced in PBMCs in response to IFN-γ treatment.

Functional activity of the purified IFN-γ–binding antibody as determined by induction of HLA class II expression

In order to confirm that the inhibition of IFN-γ–responsive genes by the patients' antibodies also corresponded to changes in induced protein expression, we studied the changes in HLA class II expression induced in PBMCs in the presence or absence of the anti–IFN-γ antibody. As shown in Figure 5, upregulation of HLA class II expression was inhibited by the anti–IFN-γ antibody, which was not observed with the nonspecific antibody on the leukocyte population. The baseline mean fluorescence index of unstimulated cells was 585 ± 9 for HLA-DR and 3 ± 0.1 for the isotope control, IgG2a antibody. The addition of IFN-γ at 1 ng/ml resulted in an increase in the mean fluorescence intensity to 2,372 ± 62 for HLA-DR expression as compared with baseline. The mean fluorescence intensity for HLA-DR in the cells with antibody alone with 1:10 dilution was 857, which indicated some nonspecific upregulation of HLA-DR expression. However, addition of the same dilution of antibody to the IFN-γ–stimulated cells resulted in a mean fluorescence intensity of only 1,236, which indicated significant inhibition of the induction due to IFN-γ ($P = 0.0002$).

Discussion

Severe, progressive, and disseminated mycobacterial infections are well recognized in association with AIDS, during immunosuppressive treatment and in patients with inherited immunological defects such as severe combined immunodeficiency. More recently, the identification of patients with genetic defects in the IFN-γ/IL-12 pathway who show a unique susceptibility to mycobacteria has provided conclusive evidence that IFN-γ plays a key role in containing intracellular infections and has explained the occurrence of unusual mycobacterial infections in patients who do not have recognizable defects in their cellular immunity (20, 21).

In this report, we have described a novel mechanism underlying a newly identified clinical syndrome of progressive mycobacterial infection in patients who have no previously recognizable immunological disorder. The clinical features of our patients are similar to those with genetic defects in the IFN-γ/IL-12 pathway in that they present with progressive or disseminated infection with mycobacteria of low virulence. The insidious, slowly progressive nature of the infection and the nonspecific presenting features including weight loss, fevers, and bone pain resulted in considerable diagnostic difficulty in each case. As in the case of patients with genetic defects in the Th1-type cytokine pathways, response to antimicrobial treatment was slow and incomplete, even when the mycobacterial infection was recognized and appropriate treatment instituted.

All 3 patients have been shown to have high titers of autoantibodies that specifically bind to IFN-γ and inhibit its ability to activate macrophage function.

Studied in serum-free medium, PBMCs from all patients showed detectable IFN-γ production in response to PHA or PMA/ionomycin. There may be several explanations for the fact that IFN-γ production by the patients' PBMCs in the absence of their serum was reduced compared with that by control PBMCs. First, complete removal of patient plasma may not have been achieved during recovery of the cells, and in view of the high titers at which the antibody can impair IFN-γ recovery, even traces of plasma may have blocked full IFN-γ recovery. Second, our previous studies of patients with IFN-γ receptor deficiency have shown reduced IFN production in response to PHA in a patient unable to respond to IFN-γ due to absence of the IFN-γ receptor (15).

The likely explanation for this observation is that IFN-γ regulates the level of its own production, in some form of feedback loop. Patients with anti–IFN-γ antibodies may have a similar defect in this feedback loop and thus resemble patients with IFN-γ receptor deficiency in this immunological phenotype. In contrast, in the presence of their own plasma, IFN-γ is undetectable, and functional IFN-γ responses are markedly impaired. We have confirmed the functional activity of the anti–IFN-γ antibody in inhibiting IFN-γ responses using 3 different methods: (a) treatment with

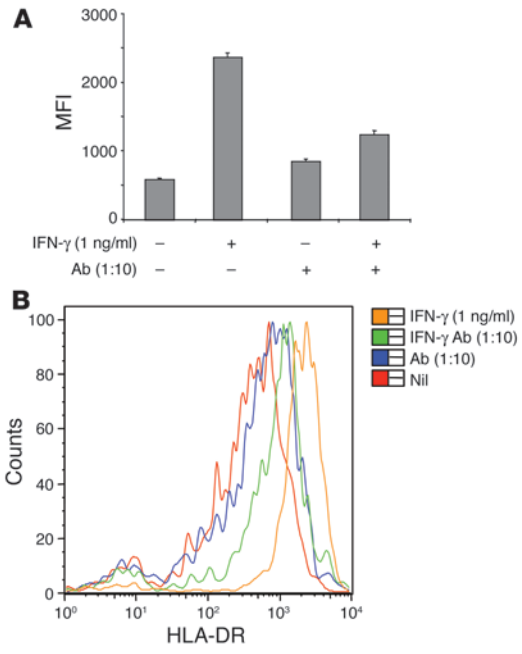

Figure 5
Inhibition of HLA class II expression by IFN-γ–binding antibody. The antibody isolated from the serum of the patient is able to neutralize the function of IFN-γ: a 1:10 dilution of the antibody in the cell culture decreases the effect of IFN-γ in upregulating HLA-DR expression on monocytes. (**A**) Results expressed as mean fluorescence index (MFI) of HLA-DR on the surface of the monocyte population, based on triplicate measurements. (**B**) Representative plot defining HLA-DR positivity. A monocyte gate was applied to the PBMCs stained for HLA-DR.

Table 1
List of genes significantly differentially expressed between PBMCs exposed to IFN-γ alone and those treated with IFN-γ and the patient anti–IFN-γ antibody

Gene ID	Full name	Mean fold change
UBD	*Ubiquitin D*	–171.23
MIG	*Chemokine (C-X-C motif) ligand 9*	–17.94
FCGR1A	*Fc fragment of IgG, high-affinity Ia, receptor for (CD64)*	–15.45
SERPING1	*Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor)*	–14.49
SCYB10	*Chemokine (C-X-C motif) ligand 10*	–14.25
WARS	*Tryptophanyl-tRNA synthetase*	–13.98
SRM300	*Serine/arginine repetitive matrix 2*	–12.99
GBP1	*Guanylate binding protein 1, interferon-inducible, 67 kD*	–10.49
PIK3C3	*Phosphoinositide-3-kinase, class 3*	–7.55
LOC51056	*pepA, cytosol aminopeptidase*	–5.74
TNFRSF5	*CD40, CD40 antigen (TNF receptor superfamily member 5)*	–5.45
EPB41	*Erythrocyte membrane protein band 4.1*	–4.74
RAB11A	*RAB11A, member RAS oncogene family*	–4.72
TNFSF10	*TNF (ligand) superfamily, member 10*	–4.56
CD4	*CD4 antigen (p55)*	–4.42
TAP1	*Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)*	–4.37
ICSBP1	*IRF8, Interferon regulatory factor 8*	–4.31
BRCA1	*Breast cancer 1, early onset*	–4.14
GLS	*Glutaminase*	–3.98
SCYA8	*CCL8, chemokine (C-C motif) ligand 8*	–3.97
STAT1	*Signal transducer and activator of transcription 1*	–3.83
ATF3	*Activating transcription factor 3*	–3.78
GBP2	*Guanylate binding protein 2, interferon-inducible*	–3.77
RPC	*RNA 3′-terminal phosphate cyclase*	–3.76
TBX21	*T-box 21*	–3.72
MRPL23	*Mitochondrial ribosomal protein L23*	–3.60
SSI-1	*SOCS1, suppressor of cytokine signaling 1*	–3.59
INHBA	*Inhibin, beta A (activin A, activin AB alpha polypeptide)*	–3.54
IRF4	*Interferon regulatory factor 4*	–3.49
SEPX1	*Selenoprotein X, 1*	–3.44
IRF1	*Interferon regulatory factor 1*	–3.42
VAMP5	*Vesicle-associated membrane protein 5 (myobrevin)*	–3.39
UBE2L6	*Ubiquitin-conjugating enzyme E2L 6*	–3.39
GLB1	*Galactosidase, beta 1*	–3.38
TXN	*Thioredoxin*	–3.28
RPS9	*Ribosomal protein S9*	–3.26
HM74	*GPR109B, G protein–coupled receptor 109B*	–3.23
CHK	*CHKA, Choline kinase alpha*	–3.17
CASP10	*Caspase 10, apoptosis-related cysteine protease*	–3.14
KLF4	*Kruppel-like factor 4 (gut)*	–3.13
PSMB8	*Proteasome (prosome, macropain) subunit, beta type, 8*	–3.12
RALB	*v-ral simian leukemia viral oncogene homolog B*	–3.11
MNAT1	*Menage a trois 1 (CAK assembly factor)*	–3.07
UBE3A	*Ubiquitin protein ligase E3A*	–3.07
LIMK2	*LIM domain kinase 2*	–3.07
H4FG	*H4 histone family, member G*	–3.03
TARBP1	*TAR (HIV) RNA-binding protein 1*	–3.02
MHC2TA	*MHC class II transactivator*	–2.99
CCNA1	*Cyclin A1*	–2.89
CD69	*CD69 antigen (p60, early T cell activation antigen)*	–2.88
RPS6KA5	*Ribosomal protein S6 kinase, 90 kD, polypeptide 5*	–2.87
IL2RA	*Interleukin 2 receptor, alpha*	–2.82
UBE1L	*Ubiquitin-activating enzyme E1-like*	–2.80
ICAM1	*Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor*	–2.80
AIM2	*Absent in melanoma 2*	–2.75
RGS1	*Regulator of G-protein signaling 1*	–2.73
DNAM-1	*CD226, CD226 antigen*	–2.67
XAP4	*HBV-associated factor*	–2.67
CSF2RB	*Colony stimulating factor 2 receptor, beta*	–2.64
CISH	*Cytokine inducible SH2-containing protein*	–2.60
APOL3	*Apolipoprotein L, 3*	–2.58
CDKN1A	*Cyclin-dependent kinase inhibitor 1A (p21, Cip1)*	–2.56
PSTPIP2	*Proline-serine-threonine phosphatase interacting protein 2*	–2.53
IFI35	*Interferon-induced protein 35*	–2.51

SAM was used to determine genes significantly differentially expressed between PBMCs exposed to IFN-γ alone and those treated with IFN-γ and the patient anti–IFN-γ antibody. A total of 120 genes was identified at a false discovery rate of less than 1 (of the median) with a delta value of 0.9243. Only genes downregulated by at least 2.5-fold in samples treated with IFN-γ and patient antibody were considered to be underexpressed. The magnitude of underexpression is marked as mean fold change. Multiple array elements of the same gene, unknown genes, and genes coding for hypothetical proteins (22) have been removed from this table. The remaining 64 unique genes are ranked by fold change.

endotoxin, which impaired TNF-α production in the presence of the patients' serum; (b) cDNA microarray analysis, which showed that a large number of IFN-γ–responsive genes were blocked in the presence of purified antibody; and (c) induction of HLA class II upregulation, which inhibited upregulation of IFN-γ.

These results establish definitively that these IFN-γ antibodies are functional in blocking the extensive cellular changes induced by IFN-γ. Furthermore, the gene expression studies add to the existing knowledge about genes that are IFN-γ responsive (23) and provide an indication of the global immune dysfunction that might occur in the presence of IFN-γ–blocking agents. It is not surprising, therefore, that the antibodies appear to produce an immunological phenotype similar to that caused by genetically determined mutations in the IFN-γ receptor genes (11, 14, 15, 20, 21, 24). The observation that inhibition of IFN-γ by autoantibodies produces marked susceptibility to mycobacteria further confirms the importance of IFN-γ in determining resistance to mycobacterial infection in humans.

The microarray data also showed an effect on the TNF-α signature; expression of the gene encoding TNF-α was downregulated over time in the presence of the anti–IFN-γ antibody. The role of TNF-α and its inhibition in mycobacterial infection is of obvious importance, and our data confirm the close interaction between IFN-γ and TNF-α, which are known to be implicated in the containment of mycobacteria and granuloma formation (25). It is possible that the changes in TNF-α signal represent a possible mechanism of refractory infection, but we interpret these changes as a consequence of the impaired IFN-γ signal rather than its cause.

Since the first presentation of patient 1 described herein, there have been reports of 2 additional patients with aggressive mycobacterial infection in whom anti–IFN-γ antibodies have been documented (26, 27). Results of our comprehensive study of the functional effects of the anti–IFN-γ antibody in blocking IFN-γ–induced genes together with those of the 2 additional reports suggest a novel mechanism of immune dysfunction and susceptibility to mycobacteria.

The major questions arising from these cases are why an autoantibody against this critical cytokine has developed and whether this mechanism accounts for susceptibility in other patients with disseminated intracellular infections. The fact that in a relatively short period 5 patients of different origin and ethnicity have been reported suggests that this disorder may be relatively common.

Anti-cytokine autoantibodies have been reported in a number of other human infectious or inflammatory conditions (28), including viral infections (29), HIV (30), and EBV (31). The autoantibodies may arise through cross-reactivity between infectious agents and host proteins; through exposure to bacterial production of novel epitopes during the interaction of human antigens and those of the pathogen; or due to a failure in self tolerance. A number of viruses including EBV are known to encode proteins with homology to human cytokines. EBV encodes a viral homolog of IL-10 that is believed to be responsible for eliciting autoantibodies against IL-10 (31). We have preliminary evidence that the purified anti–IFN-γ antibodies from our patients do recognize mycobacterial antigens, but further studies are required to establish whether the antibodies do indeed cross-react with mycobacterial proteins.

Anti–IFN-γ antibodies have been reported previously in humans. Turano et al. (32) reported the presence of anti–IFN-γ antibodies in normal individuals that interfered with Fc receptor and HLA-DR expression but did not block antiviral activity. Madariaga et al. (33) reported the presence of antibodies to IFN-γ in patients with *M. tuberculosis* infection, but their functional significance was not established. In their study, the presence of antibody did not prevent the detection of IFN-γ in the plasma. This is in contrast to the finding in our patients, where the presence of antibody blocked both the recovery and function of IFN-γ. Further studies are required to establish whether other patients with disseminated mycobacterial disease, not explained by known immunodeficiencies, have IFN-γ autoantibodies similar to those observed in our patients. In our own experiments, we have not detected impaired recovery of IFN-γ from plasma of patients with typical TB.

Our results support the hypothesis that susceptibility to intracellular pathogens in humans might originate from a range of different acquired or inborn defects in macrophage activation by IFN-γ. Autoantibody-mediated defects in this pathway represent a novel immunological defect in the handling of mycobacteria and should be considered in the investigations of patients with persistent or unusual mycobacterial infection.

Methods

Routine immunological investigations. All 3 patients were extensively investigated to exclude common cellular or humoral immunological defects. Results of tests for HIV antibodies were negative and the numbers of T cells and subclasses normal. Results of the reduction of nitroblue tetrazolium reduction test were normal; C3, C4, and total hemolytic complement levels were normal; results of tests for antibodies against nuclear antigens and DNA, rheumatoid factor, antineutrophil cytoplasmic antibodies, and antiphospholipid antibodies were negative. Immune responses to vaccine antigens were normal. IFN-γ receptor deficiency and IL-12 and its receptor deficiency were excluded, as described previously (3, 4, 34).

Experimental investigations. All blood samples from patients and controls were taken with informed consent and approval of the Clinical Ethics Committees of St. Mary's Hospital (London, United Kingdom), the Northwick Park Hospital, and the Groote Schuur Hospital. Whole blood was collected into sterile bottles containing either 10 U/ml of preservative-free heparin or no anticoagulants. Plasma was obtained by centrifugation of heparinized venous blood; serum was obtained by centrifugation of clotted blood samples. PBMCs were obtained over a Ficoll-Hypaque gradient, using standard protocols.

The 3 individuals with severe NTM infection reported in this article are referred to as patients. Healthy adult individuals, whose blood/PBMCs were used as controls in the functional assays described below, are referred to as controls.

Plasma from 3 additional groups of subjects — (a) 13 subjects with active TB; (b) 8 subjects with LTBI; and (c) 11 tuberculin-negative healthy subjects — was used to establish the specificity of the findings for patients with NTM infection, as described below.

Production of IFN-γ in whole blood and PBMCs. The production of IFN-γ in response to the mitogen PHA (Sigma-Aldrich) or PMA and ionomycin was assessed in heparinized whole blood diluted 1:10 with RPMI tissue culture medium (Invitrogen Corp.) as described previously (35). PHA (10 μg/ml) was added to aliquots of blood and incubated for 3 hours at 37°C. The concentration of IFN-γ was measured in the supernatant by ELISA using antibody pairs from BD Biosciences — Pharmingen. For assessment of IFN-γ production in the absence of plasma, PBMCs prepared from patients' or control blood were plated out at a concentration of 1×10^6/ml in RPMI culture medium and stimulated with 10 μg/ml PHA for 3 days. The supernatant was recovered, and the concentration of IFN-γ determined as described above.

Effect of patients' plasma on IFN-γ–induced macrophage activation. The ability of IFN-γ to upregulate macrophage TNF-α production in response to

endotoxin was assessed as described previously (2, 15). PBMCs from patients or healthy controls were recovered from freshly drawn heparinized blood by Ficoll-Hypaque gradient separation and plated out at a concentration of 1×10^6/ml in the presence of 10% patient or control plasma. The concentration of TNF-α in the supernatant was determined by ELISA after stimulation for 3 hours with *E. coli* endotoxin (0.2 ng/ml; Sigma-Aldrich) in the presence of concentrations of IFN-γ ranging from 0 to 10,000 ng/ml for 3 hours.

Detection of IFN-γ–neutralizing factor. Exogenous IFN-γ was added to patients' or control plasma, or separated fractions of plasma, in concentrations ranging from 0 to 10,000 ng/ml. After samples were mixed and incubated for 1 hour, the concentration of recovered IFN-γ in the plasma was determined by ELISA.

Determination of serum anti–IFN-γ IgG and IgM titers by ELISA. ELISA plates were coated with anti-human IFN-γ antibody (554548; BD Biosciences — Pharmingen) at 2 μg/ml in carbonate buffer (pH 8.2) overnight at 4°C. After washing with PBS containing 0.1% Tween-20, wells were blocked with PBS containing 10% FCS. Following washing, the IFN-γ–binding sites were saturated with 5 μg/ml IFN-γ during overnight incubation at 4°C. Control wells were not treated with IFN-γ. Plates were washed again, and serially diluted serum samples (dilutions: 1:32, 1:64, 1:128, 1:256, 1:1,024) were added in duplicate to the IFN-γ–treated and untreated wells. Following overnight incubation at 4°C, plates were washed, and peroxidase-conjugated anti-IgG antibody (A6029; Sigma-Aldrich) or anti-IgM antibody (A6907; Sigma-Aldrich) was added at dilutions of 1:2,000 (anti-IgG) or 1:3,000 (anti-IgM) for 1 hour at room temperature. After extensive washing, wells were developed using tetramethylbenzidine substrate (P4922; Sigma-Aldrich), and ODs were read at 450 nm. For determination of the anti–IFN-γ IgG or IgM titers, the mean OD values in the IFN-γ–untreated control wells were subtracted from the mean OD values in the IFN-γ–treated wells for each sample at each dilution.

Purification of the IFN-γ–binding factor. An IFN-γ affinity column was prepared by incubating 0.5 mg of IFN-γ with activated Sepharose beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. After unbound IFN-γ was removed and the column was equilibrated to pH 7 in PBS, 1 ml of either patient or control serum diluted 1:2 in PBS was passed over the column. After unbound plasma was washed off with 2 column volumes of PBS, the bound factors were sequentially eluted with 0.5 M, 1 M, and 2 M NaCl, followed by 0.1 M glycine HCl (pH 3). The eluate recovered with each step was dialyzed against PBS, concentrated to the original volume, and tested for the ability to neutralize IFN-γ as described above. The identity of the IFN-γ–binding factor was established by SDS-PAGE, N-terminal amino acid sequencing (performed at the Department of Biochemistry, University of Newcastle), and peptide mapping performed by SELDI mass spectrometry (36).

Confirmation of function of the anti–IFN-γ antibody through the use of high-density cDNA microarrays. In order to confirm that the IFN-γ autoantibodies found in our patients were functional in blocking IFN-γ–induced effects, we studied the genes in PBMCs, whose expression was altered by IFN-γ in the presence of affinity-purified anti–IFN-γ antibodies from patient 1. PBMCs from a healthy donor were plated at a density of 4×10^6/ml in RPMI culture medium and exposed to IFN-γ at a concentration of 1 ng/ml, or an equivalent volume of PBS, in the presence or absence of either purified anti–IFN-γ antibody or a nonspecific antibody (isotype control IgG2a).

At sequential time points from 0–21 hours, RNA was extracted using a standard phenol chloroform isopropanol extraction technique. The RNA was reverse-transcribed to cDNA and amplified using a modified Eberwine technique (MessageAmp aRNA kit; Ambion Inc.) as described previously (37–39). The RNA-derived cDNA sample was labeled with Cy5-dUTP and combined in equal quantity with a similarly labeled reference cDNA (Cy3-dUTP), which had been derived from a pool of RNA from a panel of 11 human cell lines (Universal Human Reference RNA; Stratagene). The probe was washed, concentrated, and competitively hybridized to custom-printed cDNA microarrays containing 37,632 elements for approximately 18,000 unique human genes. The slides were scanned using a GenePix 4000A microarray scanner (Axon Instruments). Areas of the array with blemishes or of poor quality were flagged and excluded from analysis (GenePix Pro 5.0; Axon Instruments). Using the Stanford MicroArray Database (http://genome-www5.stanford.edu//), we filtered data to include only cDNA elements with fluorescence intensities of at least 2.5-fold over background in both Cy3 and Cy5 channels and that were present on at least 80% of the arrays. The remaining gene expression ratios were normalized and the data zero transformed using a custom-designed Microsoft Excel macro (C. Liu, http://genetics.stanford.edu/~cliu/DataProc_Template_v1_2 8.xls). The transformed data were hierarchically clustered using Cluster version 2.11 and the results displayed using Treeview software version 1.60 (http://rana.lbl.gov/EisenSoftware.htm) (40).

The hypergeometric distribution was used to determine whether genes known to be induced by IFN-γ were significantly enriched in the subset of genes induced by IFN-γ and whether those genes were then downregulated in response to treatment with the purified anti–IFN-γ antibody. The hypergeometric *p* value was calculated (41) using the following formula:

Equation 1

$$p(x, N, A, n) = 1 - \sum_{i=0}^{x-1} \frac{\binom{A}{i}\binom{N-A}{n-i}}{\binom{N}{n}}, \text{ where } \binom{N}{n} = N!/n!\,(N-n)!$$

where N is the total number of named genes in the filtered population; A is the number of genes known to be induced by IFN-γ in the filtered population (22, 23), x is the number of named genes previously identified as induced by IFN-γ that were upregulated in response to IFN-γ treatment and repressed by treatment with the purified anti–IFN-γ antibody; and n is the total number of known genes induced in response to IFN-γ and repressed by treatment with the purified anti–IFN-γ antibody.

In addition, the statistical package SAM (version 1.15; http://www-stat.stanford.edu/~tibs/SAM/) was used to identify genes significantly differently expressed in the normalized data sets (42).

Confirmation of function of the anti–IFN-γ antibody by induction of HLA class II expression. IFN-γ upregulates the expression of HLA-DR on leucocytes. We utilized this upregulation to assess the ability of the anti–IFN-γ antibody to neutralize this functional effect of IFN-γ. PBMCs from a healthy human donor were plated in a 96-well tissue culture plate in triplicate at a density of 10^5 cells per well. Serial dilutions of the affinity-purified anti–IFN-γ antibody were added to the cells. IFN-γ was added to the wells at a concentration of 1 ng/ml. Control wells containing cells with IFN-γ and a nonspecific antibody; cells without IFN-γ but with either anti–IFN-γ antibody or nonspecific antibody alone; or unstimulated cells without antibody were also included. The plate was incubated overnight in a CO_2 incubator. The cells were then stained for HLA-DR expression using commercially available conjugate antibodies and analyzed by FACS (FACSCalibur Flow Cytometer; BD Biosciences) with CELLQuest software (BD Biosciences).

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