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Intractable mycobacterial infections associated with genetic defects in the receptor for interferon gamma: what does this tell us about immunity to mycobacteria?

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Introductory articles

Interferon-γ-receptor deficiency in an infant with fatal Bacille Calmette-Guérin infection

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The attenuated strain of Mycobacterium bovis *bacille Calmette-Guérin (BCG) is the most widely used vaccine in the world. In most children, inoculation of live BCG vaccine is harmless although it occasionally leads to a benign regional adenitis. In rare cases, however, vaccination causes disseminated BCG infection, which may be lethal. Impaired immunity of the host is generally thought to be the pathogenic mechanism. Disseminated BCG infection has been reported in children with inherited immune disorders. Most of these children had severe combined immunodeficiency, which is characterized by an absence of T cells, and some had chronic granulomatous disease, which is marked by an impairment of the phagocyte respiratory burst. Rare cases of BCG infection have also been reported in association with the acquired immunodeficiency syndrome. We examined the five genes coding for interferon-γ, interferon-γR1, IRF1, TNF-α, and TNF-αR1 in a child with fatal idiopathic disseminated BCG infection. We found a mutation of the gene for interferon-γR1. There was no detectable interferon-γR1 on the cells from the affected child. These findings provide further evidence of the importance of interferon-γ in the response to mycobacterial infection.* (*N Engl J Med* 1996;**335**:1956–61)

A mutation in the interferon-γ-receptor gene and susceptibility to mycobacterial infection

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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)

Tuberculosis has become a global emergency. One of the most important barriers to progress in the management of tuberculosis is our lack of understanding of the immune mechanisms that control the growth of mycobacteria in man. It is generally believed that Th1 lymphocytes are important, but the evidence is circumstantial or extrapolated from rodents, and we have almost no convincing data on the final effector pathways that kill the organisms. A fruitful approach to discovering how mycobacteria are controlled in mice has been to study the course of infection in animals modified by the administration of neutralising antibodies or by gene knockout to induce temporary or permanent deficiency of a single cytokine or cytokine receptor. The introductory articles comprise two recent studies of children with intractable mycobacterial infections but no recognisable immunodeficiency syndrome, which have revealed genetic "experiments of nature" resulting in failure to express receptors for interferon gamma (IFNγ).[1 2] It is now therefore certain that these receptors are fundamental to immunity to some members of this genus of bacteria in man. These are fascinating papers and represent a step forward in our understanding, but they also raise a whole series of questions about possible differences between rodent and human immune systems, and about the effector mechanisms of anti-mycobacterial immunity.

The patients investigated

Vaccination with Bacille Calmette-Guérin (BCG), an avirulent derivative of the organism responsible for bovine tuberculosis, occasionally causes disseminated infection.[3] In some of the affected individuals there is massive proliferation of the organisms with little evidence of a local T cell mediated reaction, suggesting a very fundamental defect in immune competence. Jouanguy and colleagues studied such a case.[1] She was a Tunisian girl who had been given Pasteur BCG at one month of age. By 2.5 months she had fever and regional adenitis. She then developed hepatosplenomegaly, granulomatous dermatitis, pneumonitis, and multiple osteolytic lesions. Biopsy specimens revealed poorly circumscribed areas of macrophages filled with acid fast bacilli subsequently identified as BCG. There were no associated epithelioid cells, giant cells, or lymphocytes. Despite appropriate chemotherapy and treatment with IFNγ she died at 10 months. The first lesson we learn from this is that even optimal chemotherapy to which the mycobacteria are fully sensitive is ineffective without help from the immune system.

The second study[2] involved four children from the same small town in Malta who presented with disseminated mycobacterial infections.[4] The mycobacterial species isolated were *M fortuitum*, *M avium* (two strains) and *M chelonei*. One child also had prolonged salmonellosis. Three of the children had died at the time of publication.

Elucidation of the genetic defect

The strategy used to investigate these children was based on the hypothesis that the deficiency was due to homozygosity for an abnormal recessive gene. The parents of the Tunisian girl were first cousins, and three of the Maltese children were known to be related while the fourth came from the same small town where intermarriage between families is common. It was also noted that the pattern of disease was reminiscent of that seen in mice with deletions (or neutralisation) of any one of the following proteins: tumour necrosis factor alpha (TNFα), TNFα receptor 1(TNFαR1), interferon gamma (IFNγ), IFNγ receptor 1 (IFNγR1), or interferon gamma regulatory factor (IRF-1).[5–9] The authors therefore determined whether the regions of the chromosomes known to contain these genes were homozygous using fresh cells or "immortalised" cell lines previously transformed with Epstein-Barr virus (EBV) as sources of DNA. Samples were also obtained from the parents and unaffected siblings. In fact, a whole genome search was used for the Maltese children using highly polymorphic probes to scan the entire genome for regions of homozygosity.[2] It is remarkable that the availability of such probes has made it possible to map the location of a rare recessive gene where only three affected patients were available. The results showed that the region encoding IFNγR1 (amongst many other genes) was homozygous in all affected children but not in the unaffected family members. Detailed analysis of the IFNγR1 gene was therefore carried out and this confirmed the presence of an abnormality.[2] Meanwhile the group studying the Tunisian child had been using a candidate gene approach, analysing only those genes considered likely to be critical for mycobacterial immunity.[1] Discussions between the two groups led to the focusing of attention on the IFNγR1 gene because there were striking clinical similarities between the Maltese children and this particular case of disseminated BCG infection. The gene turned out to be abnormal in the Tunisian child as well, though interestingly, at the molecular level, the defect in the Tunisian child was different from that found in the Maltese families.[1]

The gene for IFNγR1 from the Tunisian child had a single nucleotide deletion that resulted in the creation of a premature stop codon near the N-terminus, in a region that encodes the extracellular domain of the receptor, so the receptor would probably be non-functional. In fact, we do not know if this abnormal receptor would have retained any functionality. There was very little mRNA derived from this gene, suggesting that the abnormal transcript was rapidly degraded. No expression of IFNγR1 could be detected with monoclonal antibodies on the EBV-transformed cell line from this child, and ^{125}I-IFNγ failed to bind.

In contrast, the Maltese children had a single nucleotide substitution (A for C) rather than a deletion.[2] It allowed normal levels of expression of the mRNA, but introduced a premature stop codon so that the resultant protein was truncated with no membrane or intracellular domain. The cells of these patients may therefore conceivably release truncated extracellular domains but, as expected, flow cytometric analysis with monoclonal antibodies to IFNγR1 failed to show any membrane-associated receptor, while the parents' cells (heterozygous) had intermediate levels of expression. Expression remained undetectable after incubation of the patients' cells with dexamethasone, which increases still further the membrane expression of IFNγR1 on normal cells.

Is the role of IFNγ in man different from its role in mice?

The pattern of disease susceptibility resulting from loss of competent receptors for IFNγ ought to provide some clues as to the mechanism of the susceptibility, but this susceptibility may be different in the two species.

In mice, knocking out the gene for IFNγ or for its receptor leads to susceptibility to a wide range of

intracellular (including *M avium*[10]) and extracellular bacteria, as well as to viruses and parasites.[1] In man the only reported effect is susceptibility to otherwise avirulent mycobacteria and, perhaps, increased susceptibility to salmonella. This difference between man and mouse may be an artefact due to the fact that in murine models these susceptibilities have been demonstrated by laboratory workers with syringes, often administering doses that would not normally be encountered, in order to obtain high rates of infection. It is not possible to predict what would happen if such an experiment were performed on IFNγR-deficient children. It would be rash to assume that they would not become infected with a similarly wide range of organisms. It is noteworthy that the infections seen in these five children were all caused by "atypical" mycobacteria that are ubiquitous in the environment. Would they have proved to be abnormally susceptible to *M tuberculosis* if they had encountered it? It is tempting to assume that they would, but in fact treatment of *M avium* infection with IFNγ has been successful in man,[11] whereas this cytokine is not helpful in human tuberculosis. We do not know whether this is because IFNγ is unimportant in human tuberculosis, or because plenty is already being produced so that IFNγ is not the limiting factor in the immune response of patients with tuberculosis.

Although we cannot know whether these children had been exposed to *M tuberculosis*, it is inconceivable that they had not been exposed to organisms such as herpes viruses that are thought to require Th1-mediated immunity. It is therefore tempting to hypothesise that there may be a real difference between mice and men in the role of IFNγ, which may be more "mycobacterium-specific" in man. An extreme view (likely to be wrong because it is so extreme) would be that mycobacteria have posed such a great threat during human evolution (and indeed continue to kill 10 million of us each year) that the selection pressure induced by mycobacteria has caused human IFNγ to become a specialised anti-mycobacterial cytokine.

Interpretation of the relevance of the mouse data to man is further complicated by the fact that mechanisms required for the clearance of acute infections may differ from those required for clearance of established ones. For instance, mice with disrupted IFNγ genes can mount an effective cytotoxic T cell response against an acute challenge with lymphocytic choriomeningitis virus and actually clear the virus.[12] On the other hand, IFNγ is essential for the clearance of chronic persistent infection which more closely mimics the situation in these children.

There is therefore some reason to suppose that IFNγ plays a different role in man and mouse. This is definitely not conclusive but, if correct, we might expect to find that the crucial mechanism leading to selective susceptibility to mycobacteria in these children is regulated differently in the two species.

What it all means for IFNγ function

What can we deduce from these findings? IFNγR1 is ubiquitously expressed on cells other than erythrocytes. The extraordinary susceptibility to infection with mycobacteria of low virulence could, strictly speaking, therefore be due to the functional failure of some unidentified accessory cell required, for instance, for the formation of granulomas. Even if we assume that the defect exerts its critical influence within the macrophage, there are still numerous possibilities. Macrophages play a crucial role at several different points within the pathway of cell-mediated (Th1-mediated) immunity, and IFNγ regulates all of these roles (fig 1).

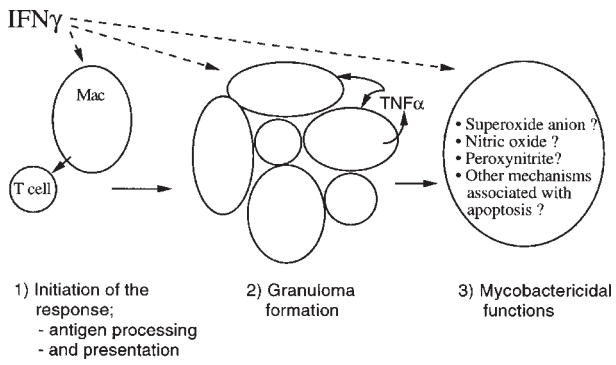

Figure 1 Interferon gamma receptors are ubiquitously expressed. Even if we consider only the macrophage, IFNγ regulates multiple functions at different stages of the cell-mediated immune response. The affected children do initiate Th1 responses, but there are no granulomas and the mycobacteria are not killed. The problem could lie anywhere within stages 2 and 3.

ANTIGEN PRESENTATION, INITIATION OF THE IMMUNE RESPONSE

First, macrophages are important antigen-presenting cells involved in the initiation of the response. IFNγ upregulates expression of the major histocompatibility complex on macrophage membranes, and these glycoproteins present antigen-derived peptides to T lymphocytes. However, there does not appear to be a defect in response initiation in these children. The tuberculin-driven T cell proliferation was normal in the Tunisian girl and, still more striking, she had a positive tuberculin reaction (16 mm induration) to 10 IU of purified protein derivative (PPD).[1] The absence of IFNγR1 therefore clearly does not block initiation of a Th1 response. By themselves these findings do not eliminate the possibility that regulation of MHC expression is the problem in these patients. It is possible that the response is initiated by other antigen-presenting cells – for example, dendritic cells – but that the Th1 lymphocytes generated cannot "see" the infected macrophages.[13] In fact, mice that lack IFNγR1 show decreased expression of MHC class II molecules on macrophages following systemic infection with BCG.[14]

However, disseminated BCG infection was not reported in children with a complete deficiency of MHC class II molecules[15] so this hypothesis is therefore unlikely.

GRANULOMA FORMATION AND ENHANCED RELEASE OF TNFα

The next phase in the development of immunity to mycobacteria is the formation of granulomas. It seems that in mice TNFα is needed for this to occur.[16] Here again we can blame the lack of macrophage activation by IFNγ, because exposure to IFNγ strikingly increases the ability of appropriately triggered macrophages to release TNFα. Indeed, the failure of IFNγ to enhance endotoxin-triggered TNFα release from the macrophages of these patients was one of the first immunological abnormalities detected.[4] Unfortunately we do not know whether TNFα is required for granuloma formation in man, but it may be relevant that, in spite

of the presence of a Th1 response, granuloma formation was not seen in the lesions of these children.[12] This hypothesis therefore remains a possibility.

MYCOBACTERICIDAL MECHANISMS WITHIN MACROPHAGES

Finally, IFNγ may be involved in activating the pathways in macrophages that actually kill the bacteria. How are mycobacteria killed? There is evidence that, in the mouse, IFNγ activates macrophages so that they express enhanced capacity for production of oxygen reduction products such as superoxide anion and hydrogen peroxide, and also increased expression of the inducible form of nitric oxide synthase (iNOS; also known as type II NOS). TNFα may then act as a trigger for NO production. This molecule may have some intrinsic antimycobacterial activity,[17] or it may interact with oxygen reduction products to yield peroxynitrites that may be the final effector molecules.[18]

DO HUMAN MACROPHAGES MAKE HIGH OUTPUT NITRIC OXIDE AND, IF SO, UNDER WHAT CIRCUMSTANCES?

The regulation of high output NO production by human cells is poorly understood, and the promoter regions of the mouse and human iNOS genes are rather different. Simple exposure to IFNγ does not enable high output NO production by human monocytes. Interestingly, IFNγ used by itself also fails to cause inhibition of growth of *M tuberculosis* in human macrophages whereas it is very effective on murine macrophages.[19,20] For this reason, in view of the possibility that man and mouse suffer different consequences from losing IFNγ function, an abnormality of the NO pathway must be another possible explanation for the specific mycobacterial susceptibility in these children.

It was at first thought that human macrophages were unable to express iNOS, and that even if they did they would not be able to make high levels of NO due to the absence of adequate levels of tetrahydrobiopterin, an essential cofactor for iNOS function. In fact, the situation is much more complex and obscure. It now appears that human macrophages can express iNOS, detectable by antibody or reverse transcriptase polymerase chain reaction (RT-PCR), after stimulation with IFNγ and endotoxin.[21,22] Thus, IFNγ could also play a part in this pathway in man. However, NO production remained trivial even when intracellular biopterin levels were raised by incubation with a membrane permeable derivative (sepiapterin). Stimuli tried included LPS, ConA, PHA, PMA, A23817, TNFα, GM-CSF, IL-1, IL-2, IL-4, IL-7, IL-6, $1,25(OH)_2$-D_3, and combinations of these.[21] Neutralising transforming growth factor (TGF)-β was also ineffective, as was exposure to *M avium* or *M tuberculosis*. (Some reports of NO production by human macrophages infected with *M tuberculosis* have been guilty of an embarrassing error. This organism expresses a potent nitrate reductase, so it generates nitrite from nitrate. Nitrite is often quantified as a surrogate for NO release since NO rapidly decays to this anion. Experiments involving *M tuberculosis* that use the nitrite method therefore mean nothing if there is any nitrate in the medium.) However, it is claimed that high output NO production is a property of alveolar macrophages from patients with tuberculosis.[23] What these authors have actually shown is rather indirect. The alveolar macrophages certainly express iNOS (that, at least, is no longer in doubt), but they also contain diaphorase activity. These authors consider diaphorase activity to be a reliable correlate of functional high output enzyme.[23]

If human macrophages really are capable of high output NO production, what signals do they require? As already outlined, many signals, including those that are effective in the mouse, have been shown not to work on human cells, even when they lead to expression of the enzyme.[21] However, there is evidence that stimulation of human macrophages with IL-4, followed by further stimulation via the low affinity IgE receptor (CD23; FcεFRII) in the absence of IL-4, can induce high output NO.[24–26] Originally this pathway was thought to require IFNγ as well, but now it appears that it works without, though both IFNγ and TNFα enhance it. If this function of IL-4 and CD23 is confirmed we will be forced to ask whether, in human tuberculosis, immunity requires both a Th1 response (to provide IFNγ, TNFα, and granuloma formation) and a Th2 response (to provide the IL-4, CD23, and NO). Actually there is clear evidence for IgE antibody[27] and for enhanced IL-4 expression in tuberculous individuals.[28] If correct, this is totally different from the situation in mice. In the latter, even a small Th2 component greatly increases susceptibility to the disease.[29]

The apparent difference in disease susceptibility between IFNγR1-deficient mice and humans (if real) could therefore lie in the different regulation of high output NO release. Perhaps it all depends on whether the infecting organism triggers an alternative non-IFNγ-dependent pathway in the host species. In the mouse there is some evidence that other cytokines such as IFNα and IFNβ can occasionally provide such an alternative to IFNγ, even if they are less efficient than IFNγ itself.[14] Another example is the apparent ability of TNFα and IL-12 to bypass a total lack of IFNγ due to gene knockout in a murine model of *Leishmania donovani* infection and cause NO-dependent control of infection.[30] However, mice without IFNγR1 may become susceptible to a wide range of infections because few of these organisms trigger sufficiently high levels of the cytokines that can bypass the defect in NO release. In contrast, the IFNγR1 defect may be more readily

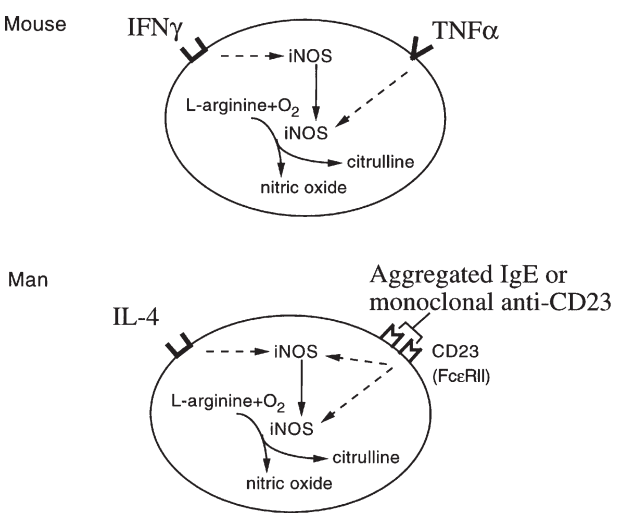

Figure 2 One possible candidate is high output nitric oxide (NO) production. The most efficient pathway for induction of high output NO in mice involves IFNγ and TNFα. Human macrophages are most convincingly triggered via a Th2-associated pathway, though both IFNγ and TNFα can augment it[24] and can probably trigger NO under some circumstances. Macrophages from children without IFNγR1 and infected with organisms that fail to evoke Th2 cytokines might lack this capability entirely. This is entirely hypothetical.

LEARNING POINTS

- **Children with inherited defects of the interferon gamma receptor (IFNγR1) have greatly increased susceptibility to infection with mycobacteria of low virulence.**
- **Children without IFNγR1 cannot be cured of disseminated BCG infection, even when given optimal chemotherapy to which the organism is fully sensitive.**
- **In man this defect may lead to specific susceptibility to this group of organisms; genetically modified mice that lack IFNγ or IFNγR1 are susceptible to a wide range of bacteria, viruses and parasites.**
- **IFNγR1 is expressed on most (perhaps all) nucleated cells so the critical defect is difficult to identify.**
- **Affected children still develop Th1 responses and have positive lymphocyte proliferation and skin test responses to tuberculin, but granulomas do not form.**
- **Likely candidate sites for the crucial defect include granuloma formation and high output nitric oxide secretion, the control of which differs markedly between man and mouse.**

bypassed in man if many organisms tend to trigger the IL-4/CD23-dependent mechanism of NO release. Thus, humans without IFNγR1 may become susceptible only to organisms that fail to evoke this alternative pathway. The low virulence mycobacteria infecting these children may have this characteristic since mycobacteria are such good Th1 adjuvants. At present this is pure speculation and very far from convincing (fig 2).

ALTERNATIVE KILLING PATHWAYS THAT MAY INVOLVE IFNγ

Several other possibilities deserve brief mention. As in murine macrophages, expression of iNOS in human neutrophils can be induced by incubation with a mixture of IL-1, TNFα, and IFNγ,[31] and these cells are capable of nitration of ingested organisms. Can we rule out the neutrophil as an essential effector cell requiring an IFNγ signal in mycobacterial infections?

This suggestion and the previous hypothesis both presuppose that NO is required for immunity to mycobacteria. Actually we do not even know that this is true. Certainly, blocking iNOS with specific inhibitors will cause increased susceptibility in mice, but NO has a wide range of signalling functions and this susceptibility may not be due to direct inhibition of NO-dependent bacterial killing pathways in macrophages.

Recently there has been provocative evidence that certain signals that induce apoptosis of infected macrophages can lead to death of some of the contained mycobacteria. For instance, hydrogen peroxide simultaneously causes apoptosis and reduced viability of phagocytosed *M avium* in human monocytes.[32] A similar effect can be induced by triggering apoptosis of human macrophages infected with *M tuberculosis* via the purinergic receptors (D. S. Kumararatne, personal communication). It is too early to say whether IFNγ is crucial to the effective activation of these pathways, or even whether they are physiologically important.

Conclusions

We can now be certain that IFNγ receptors – so presumably also IFNγ itself – are particularly important for immunity to BCG and to several atypical mycobacteria of low virulence. Since this cytokine has multiple effects on essentially all nucleated cell types, we cannot yet deduce the nature of the critical mechanism or mechanisms. The most likely candidates are granuloma formation or activation of unidentified killing pathways within macrophages. A rather tenuous case can be made for involvement of high output NO release.

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