

## Pathogenesis of nerve damage in leprosy: genetic polymorphism regulates the production of TNF $\alpha$

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**Summary** Studies carried out over the last decade have strongly suggested that TNF $\alpha$  both overtly participates in the cell-mediated immune response against *Mycobacterium leprae*, and is overproduced during reaction. In addition, reactions are intimately related to the onset of nerve damage. Finally, TNF $\alpha$  has been implicated in the pathogenesis of many human and experimental autoimmune peripheral neuropathies that, as in leprosy, result in demyelination and axonal lesions. Because of recent findings associating human TNF $\alpha$  mutant alleles at the -308 position with increased production of TNF $\alpha$  in many immunological and infectious diseases, an investigation of the role of TNF2 in predisposing leprosy patients to reaction has been undertaken. Analysis of 300 patients with leprosy—210 multi-bacillary and 90 paucibacillary—has shown that the percentage of reactional patients was similar among both carriers and non-carriers of the TNF2 allele. However, a separate analysis of 57 carriers of TNF2 found that reactions occurred much more frequently among heterozygous than among homozygous patients. Moreover, the frequency of neuritis was somewhat greater among the heterozygous patients than among the non-carriers. Enhanced serum levels of TNF $\alpha$  have been noted in both TNF-1 and TNF-2 mutant patients in the course of leprosy reaction. Our observations to date suggest that other factors not related to the presence of the mutant gene may lead to the TNF $\alpha$  hyper-responsiveness observed during reaction.

### Introduction

Human TNF $\alpha$ , also known as cachetin, is a cytokine that exhibits wide-ranging biological activities and systemic effects, including protection against infection, surveillance against tumours, and stimulation of inflammatory responses, in addition to mediation of septic shock in chronic infection and cachexia in cancer patients. TNF $\alpha$  has also been shown to be capable of directly causing the destruction of myelin and oligodendrocytes *in vitro*.<sup>1</sup> Because overproduction of TNF $\alpha$  has been shown to be deleterious to the host, leading to irreversible organ failure and death, both the beneficial and negative effects of this cytokine require that stringent controls be imposed on its synthesis. Consequently, TNF $\alpha$  gene expression must be tightly regulated.<sup>2</sup> Regulation of the production of TNF $\alpha$  occurs at both the transcriptional and post-transcriptional levels; because the rate of transcription is an important regulator of

production, it has been suggested that, in certain situations, variations within the regulatory regions of the TNF $\alpha$  gene contribute to alterations of cytokine-expression. Moreover, linkage between mutations in the TNF promoter region and development of acute inflammatory complications during the course of some diseases has been clearly established, the prime example being cerebral malaria.<sup>3</sup>

It has been shown that a single base polymorphism within the promoter region may alter the rates of both gene transcription and protein production. To date, 13 different allelic variations have been found to exist in the promoter region of the TNF $\alpha$  gene. Because all of these variations appear to be located within the limits of the gene's regulatory region, determination of the association of these variations with expression of TNF $\alpha$ , as well as with disease, remains an important area of study.

A biallelic polymorphism, consisting of a G versus A transition at position -308 (TNF2) of the TNF $\alpha$  promoter, although not apparently related to any currently recognised DNA-binding protein sequence motif, appears to be directly involved in the alteration of TNF $\alpha$  gene expression.<sup>4</sup> Several studies of the role of this mutation in production of TNF $\alpha$ , although contradictory,<sup>5,6</sup> have shown, in support of a predisposing effect,<sup>4,7</sup> the TNF2 allele to be associated with higher constitutive and inducible levels of transcription compared to the wild type allele (TNF1). Moreover, a relationship between the presence of the TNF2 allele and disease has also been detected in mucocutaneous leishmaniasis,<sup>8</sup> meningococcal disease,<sup>9</sup> and lepromatous leprosy.<sup>10</sup>

Studies carried out during the last decade by our group and others have strongly suggested that: (i) TNF $\alpha$  both overtly participates in the cell-mediated immune response against *M. leprae*, and is overproduced during reaction;<sup>11,12</sup> (ii) reactions are intimately related to the onset of nerve damage,<sup>13,14</sup> and (iii) TNF $\alpha$  has been implicated in the pathogenesis of many human and experimental autoimmune peripheral neuropathies that, as is the case in leprosy, result in demyelination and axonal lesions.<sup>15,16</sup>

Because of recent findings associating the human TNF $\alpha$  mutant alleles at the -308 position (individuals carrying AA or GA instead of GG) to the increased production of TNF $\alpha$  in immunological and infectious diseases, we decided to investigate the role of TNF2 in predisposing leprosy patients to reaction and nerve damage.

## Materials and methods

To date, 300 leprosy patients classified according to Ridley and Jopling,<sup>17</sup> covering the entire clinical spectrum [210 multibacillary (MB) and 90 paucibacillary (PB) patients], and 92 controls have been typed for the -308 mutation.

Genomic DNA was prepared from frozen whole blood (300  $\mu$ l) by a commercially available DNA extraction kit (Gibco BRL, Gaithersburg, MD, USA). Typing of the TNF $\alpha$  promoter region (107 bp fragment) for analysis of polymorphisms at the -308 position was performed with the aid of specific primers through a single PCR step and further digestion with *Nco*I.<sup>20</sup>

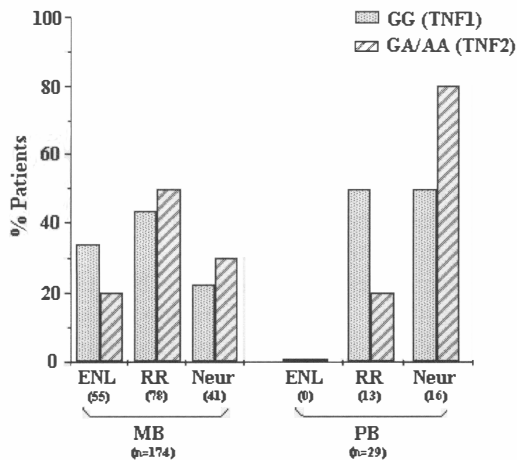
Determination of the quantity of TNF $\alpha$  protein in the sera of patients at the time leprosy was diagnosed (non-reactional patients;  $n = 33$ ) and during a reaction [either erythema nodosum leprosum (ENL), reversal reaction (RR), or neuritis;  $n = 29$ ] was performed by ELISA (Innogenetics N.V., Gent, Belgium) and processed according to the manufacturer's instructions.

## Results

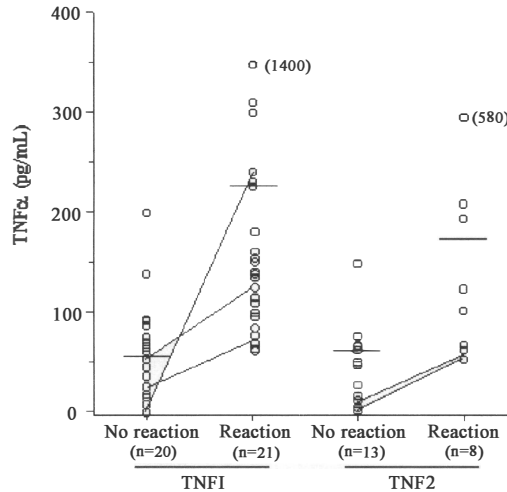
Preliminary analyses of this population showed a significantly ( $\chi^2 = 7.55$ ,  $P = 0.005$ ) higher frequency of the mutant allele among healthy individuals (16.3%) than among leprosy patients (10.8%), suggesting that the presence of the TNF2 allele protects against the development of the more severe form(s) of leprosy.<sup>18</sup> Analysis of the occurrence of reactions in this group of leprosy patients showed that the proportion of patients with reaction was similar among carriers (61.4%) and non-carriers (69%) of the TNF2 allele. There was no difference of frequency of the genotype between patients with reaction and those without. The frequency of TNF1 was a little greater among patients with ENL, whereas TNF2 was slightly more frequent among patients presenting neuritis.

The relationship of the -308 mutant allele to clinical diagnosis and type of reaction among the 203 reactional patients (174 MB and 29 PB) is shown in Figure 1. Analysis of the TNF2 carriers ( $n = 57$ ) demonstrated that reactions were much more frequent among the heterozygous (65.3%) than among the homozygous (37.5%) patients. Moreover, the frequency of neuritis was somewhat greater among the heterozygous patients than among the non-carriers (37.1% versus 26.2%, respectively).

Detection of TNF $\alpha$  protein by ELISA was assessed in the sera of the same patients, 41 of whom were TNF1 and 21 TNF2, at diagnosis and during reaction. Enhanced TNF $\alpha$  levels have been noted in both TNF-1 and TNF-2 mutant patients in the course of reaction (see Figure 2). Mean levels of TNF $\alpha$  were similar for both TNF1 and TNF2 patients in the absence of, as well as during, reaction; and both TNF1 and TNF2 patients showed increased TNF $\alpha$  levels during reaction. Thus far, our data suggest that other factors, not exclusively related to the presence of the mutant gene, may be responsible for the hyper-responsiveness to TNF $\alpha$  observed during the reaction.



**Figure 1.** Distribution of the -308 TNF $\alpha$  mutant allele according to clinical diagnosis and type of leprosy reaction. A total of 203 leprosy patients (174 MB and 29 PB) with reaction were typed as homozygous for the wild type allele (GG, non-carriers) and carriers of the mutant allele (GA and AA). ENL = erythema nodosum leprosum; RR = reversal reaction; Neur = neuritis. Numbers in parentheses indicate the number of individuals assayed in each group.



**Figure 2.** Detection of TNF $\alpha$  in the sera of TNF1 ( $n = 41$ ) and TNF2 ( $n = 21$ ) leprosy patients. A total of 33 patients without reaction and 29 patients with reaction were evaluated. The quantity of TNF $\alpha$  protein (pg/ml) in the serum samples was determined by ELISA. Symbols represent each individual patient assayed. Horizontal bars represent the mean TNF $\alpha$  levels per group of patients tested. Lines indicate the same patient analysed before and during the reaction ( $n = 5$ ).

## Discussion

We do not yet understand the pathogenesis of nerve damage in leprosy. After the invasion of Schwann cells by *M. leprae*, the molecular mechanism(s) involved in nerve injury remain unexplained. The evidence accumulated to date indicates that, in both leprosy and other demyelinating diseases, nerve injury may result from the same or similar mechanisms. The involvement of cytokines, principally TNF $\alpha$ , in the pathogenesis of peripheral neuropathies has also been widely suggested. The demonstration<sup>19</sup> that TNF $\alpha$  may induce apoptosis of Schwann cells suggest that such a mechanism could trigger demyelination and further nerve injury.

Cytokine-producing cells in peripheral nerves include resident and recruited macrophages, lymphocytes, and probably Schwann cells and neurons. Moreover, TNF $\alpha$  could contribute to myelin damage by triggering enzymes, such as neutral sphingomyelinase, within the myelin sheath, leading to changes of the lipids that are potentially destabilizing to myelin.<sup>15</sup>

This study of TNF $\alpha$  polymorphism indicates that, in addition to the primary association of the mutant allele with PB leprosy,<sup>18</sup> there may also be an association with the occurrence of neuritis, especially in the PB form of the disease. However, demonstrating an association between the TNF genotypes and the serum TNF $\alpha$  levels has proven to be a very difficult task. The identification of genetic markers for susceptibility to, or severity of, inflammatory reactions is a goal to be pursued in leprosy and several other diseases. Even so, one of the greatest challenges facing scientists today is the integration of the myriad components of the organism in such a way as to clarify its functioning as a whole, as well as that of its components. Typing of mutations in the TNF $\alpha$ , IL-10, and other genes, followed by an attempt to associate them with their functional roles *in vivo*, and the different clinical settings presented by each disease, remain important issues that require further research.

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## DISCUSSION

*Professor Smith:* From a consideration of the work presented and the ensuing discussions, I have chosen four major topics that, in my view, represent priority areas for research in the area of nerve damage and reactions. (i) I believe we need an improved understanding of the mechanisms underlying nerve damage and reactions. (ii) The second priority area is that of improved treatment of nerve damage. (iii) Third is the matter of predicting reaction and nerve

damage, and their prevention. (iv) Finally, we must standardize terms and definitions, particularly for the purpose of clinical studies.

*Professor Brennan:* I've been thinking about the nature of the bacterial stimulus to reactions and nerve damage. We have heard that one of the predictors is MB leprosy; this implies the importance, at some time in the process, of the size of the bacterial burden and the presence of bacterial products. What might these products be? One thinks of rather odd molecules that can withstand natural degradative processes, thus eliminating peptides. One such substance is lipoarabinomannan (LAM). *M. leprae* produces it in massive quantities, and mammals lack the glycosidases needed to degrade this material. We've heard from Drs Kaplan, Lockwood and Nath about the roles of certain cytokines in pathogenesis; there is some evidence that LAM stimulates production of TNF $\alpha$  and others of these cytokines. Assays exist for LAM and similar molecules. If one could demonstrate a relationship between the presence of LAM, for example, and nerve damage, then one could begin to consider means of clearing these materials.

*Dr Lockwood:* We have identified LAM in nerve biopsy specimens obtained from patients in reaction.

*Professor Britton:* One might wonder what are the metabolic effects of LAM within the Schwann cell. Once the Schwann cell has been injured, axonal damage is inevitable. The issue of metabolic effects in the Schwann cell might be a fruitful area for research.

*Dr Gupte:* I wonder if the area of surgical treatment of nerve damage has been sufficiently investigated. The literature contains some evidence of the efficacy of surgical decompression of inflamed nerves, for example.

*Professor Smith:* Do you feel that the evidence is sufficiently promising to justify further research?

*Dr Gupte:* Yes, I believe it is.

*Dr Lockwood:* I believe there is an urgent need for a trial of decompression *vs.* steroids. Many surgeons currently perform decompressions on patients being treated with steroids. A number of manuscripts have been submitted to *Leprosy Review*, in which it has been very difficult to distinguish between the effects of surgery and those of steroids.

*Dr van Brakel:* A surgical colleague who worked in Nepal in the past has already prepared a protocol for such a trial.

*Professor Ji:* Surgical decompression has been commonly used in some countries. I agree that the efficacy of decompression should be determined. However, I don't think a clinical trial is justified; I believe that all patients must first receive steroids. Perhaps, if improvement following therapy with steroids is insufficient, then one might consider decompression, among other alternatives.

*Dr Nunn:* Estimates of the burden of a disease have been quite useful in determining priorities, particularly when one must choose between diseases. Are you satisfied that the currently available methods of measuring disability resulting from leprosy are accurate? The DALY (Disability-Adjusted Life Years) score of leprosy is by far the smallest of all ten diseases included in TDR (the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases).

*Professor Smith:* I think we're quite good at measuring physical impairments, but not disabilities and handicaps and the social consequences of these.

*Dr Rambukkana:* It would be good if we could remove the organisms from the Schwann cells. Even irradiated *M. leprae* can produce considerable damage to Schwann cells. This could account for the progression of nerve damage after MDT.

*Professor Smith:* Dr Cole, you listed some of the potential applications of the new information coming from sequencing of the *M. leprae* genome. Do you see an application to the area of nerve damage?

*Dr Cole:* Yes and no. We have no clear leads to what could be causing nerve damage and what could be triggering bystander reactions. However, I think I can explain the fact, pointed out by Professor Brennan, that *M. leprae* produces 10 times the quantity of LAM that is produced by *M. tuberculosis*. The gene that regulates synthesis of arabinogalactan in *M. tuberculosis* is inactivated in *M. leprae*.

*Professor Nath:* I doubt that long-lasting bacterial products can explain the episodic nature of reactions.