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Expression and cytokine secretion in the states of immune reactivation in leprosy

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Abstract

Leprosy is a chronic inflammatory disease caused by *Mycobacterium leprae*. The human response to this pathogen exhibits intriguing aspects which are up to now not well understood. The present study discusses the probable mechanisms involved in *T* cell-specific unresponsiveness observed in lepromatous patients. Analysis of the cytokine profile either in blood leukocytes or in skin specimens taken from leprosy lesions indicates that some parameters of *Th1* immune response are present in lepromatous patients under reactional states.

Key words
- Leprosy
- Reactions
- Cytokines

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Introduction

Leprosy is a chronic inflammatory disease caused by *Mycobacterium leprae*, an obligate intracellular pathogen that provides a model to address the role of *T* cell subsets in human disease. The outcome of any infection relies on a complex cascade of interactions in vivo. The dynamic nature of the immune response to *M. leprae* is reflected in both the variety of outcomes in different subjects and the varying responses with time by one individual.

Subclinical infection with *M. leprae* occurs in a large proportion of those who are exposed to the leprosy bacillus. Nevertheless, it is estimated that *10* to *12* million people suffer from leprosy worldwide. The disease presents a broad spectrum of clinical forms which depend essentially on host immune response. At one end of the spectrum lie tuberculoid patients, who have localized skin lesions, measurable *T* cell responsiveness in vitro or in vivo, a strong delayed-type hypersensitivity response, and low levels of serum antibodies to *M. leprae*. At the other end of the spectrum are lepromatous patients with multiple and diffuse lesions and a diminished skin test response to lepromin, who are unable to limit the spread of the bacteria which, in the absence of chemotherapy, may disseminate throughout the body. These patients show little detectable in vitro proliferative or in vivo skin test responses specific for *M. leprae* antigens, although serum antibody levels specific for *M. leprae* are very high. In addition, histopathological aspects of leprosy lesions are also characteristic of patients in each group at one pole of the spectrum. Tuberculoid lesions present well-formed epithelioid granulomas consisting of *T* lymphocytes and differentiated macrophages. Bacilli are rarely found. Lepromatous lesions consist of macrophages containing

large numbers of bacteria. Numerous histochemical studies have confirmed the predominance of *CD4+* over *CD8+ T* cells in tuberculoid leprosy, while in lepromatous leprosy lesions there are far fewer lymphocytes and similar proportions of *CD4+* and *CD8+ T* cells *(1)*.

Cellular immune response in leprosy

Lepromatous leprosy patients display a selective immunological unresponsiveness to *M*ycobacterium leprae antigen with absence of delayed-type hypersensitivity, *T* cell proliferation, and deficiency in the production of growth factors such as *IL-2*. These patients also fail to produce interferon-gamma *(IFNγ)* in response to *M.* leprae, which seems to correlate with the inability of their macrophages to prevent the growth of the mycobacterium. Cellular anergy observed in lepromatous patients appears to be *M.* leprae specific since the immune response against other antigens is largely normal. Active suppression by macrophages and/or *T* cells may explain their inability to respond to leprosy bacilli. Lepromatous patients carry a high load of bacilli which may play a role in the in vivo induction of immune tolerance.

Genetic factors have been considered for a long time in leprosy. This is largely due to the observation of clustering of leprosy around certain families, and the failure to understand why certain individuals develop lepromatous leprosy while others develop the non-lepromatous form. De Vries et al. *(2)* were the first to find that *HLA* genes are important genetic factors in leprosy. It is evident now that these *HLA* genes do not determine susceptibility to leprosy per se but rather control the type of leprosy that develops upon infection of susceptible individuals. Interestingly, among the susceptible individuals, those with *HLA-DR3* more often develop tuberculoid leprosy, whereas those with another *HLA* type, *DQ1*, more often develop lepromatous leprosy *(2)*.

The crucial role of *CD4+ T* helper cells in orchestrating cell-mediated immunity has led to the concept that macrophage activation by cytokines secreted from major histocompatibility complex *(MHC)* class *II*-restricted, antigen-specific *T* helper type *I (Th1)* cells is the principal mechanism of protection against intracellular microbes. In recent years, however, this concept has undergone major revisions given the important contribution of *CD8+ T* cells to acquired resistance to at least some mycobacterial species, and the role of γδ-*T* cell receptor *(TCR)* cells in antimycobacterial immunity *(3)*. More recently, novel classes of *T* cell receptor ligands and antigen presentation molecules have entered the scene. *T* cells can also recognize non-peptide ligands such as mycobacterial mycolic acids, lipoarabinomannan and isopentenyl phosphate groups *(4)*. Moreover, peptides as well as non-peptide ligands can be presented by non-*MHC*-encoded but *MHC*-like molecules such as *CD1*. It is completely unclear, however, to what extent these novel *T* cell receptor ligands precisely contribute to protective immunity or immunopathology, and whether the results obtained in experimental infections in mice can be extrapolated to naturally occurring chronic infection in humans.

Moreover, recent studies have focused on the cytokine profiles of *M.* leprae reactive *T* cells. It has been proposed that the two polar stable forms of leprosy reflect the dominance of two major *T* cell subpopulations. The hypothesis that the spectrum of leprosy reflects the balance between *T* helper *(Th1* and *Th2)* cell populations activated by mycobacteria, defining the cytokine profile produced by each of these populations *(5)*, is indeed exciting, but the data accumulated so far have not backed it up. In tuberculoid leprosy, *Th1* cells producing both *IL-2* and *IFNγ* may lead to an efficient immune response while in lepromatous leprosy, the *Th2* population may be preferentially activated, producing both *IL-4* and *IL-5*. Studies

have provided evidence in favor of this hypothesis, whereas others have provided contrasting results.

Instability in the immune response to M. leprae

Actually, leprosy is not a stable disease and the above described polar forms of leprosy are rarely diagnosed at Clinical Units. Between these two extremes (polar forms) a continuous clinical and histopathological range, termed borderline leprosy, is more often seen. In this case, the cell-mediated immune response is not stable and patients present an increased immunological response to *M.* leprae as they approach the tuberculoid end of the spectrum.

Interestingly, we have recently demonstrated that biopsies from some unreactional borderline lepromatous patients obtained at diagnosis show the pattern of in situ activation instead of the histological characteristics of non-responders. Presence of high numbers of *CD4+ T* cells in the inflammatory infiltrate, thickening of the epidermis, and expression of *HLA-DR* and intracellular adhesion molecule *(ICAM-1)* on the surface of keratinocytes have been noted *(6)*.

At this point, the following questions demand clarification: *1)* May the pattern of immune response seen in borderline patients be the same as found in the polar forms of leprosy? *2)* Is the non-responsiveness seen in borderline lepromatous patients the result of a predominance of a *Th2* over a *Th1* response? *3)* May the cytokine profile expressed by these patients be the result of a *Th0* response? *4)* May the non-responsiveness seen in these patients be modulated by the presence of mycobacterium components, such as *LAM* and *PGL1*, known to have suppressor properties on *T* cells and macrophages?

In a very recent study *(7)*, the functional properties of *CD4+ CD8- T* cell clones isolated from the skin lesions and blood of leprosy patients across the spectrum were assayed. Substantial heterogeneity in the production of cytokines was observed. A variety of patterns of cytokine secretion distinct from those of *Th1*, *Th2* or *Th0* was evident, although no striking correlation with clinical status was apparent. Noteworthy was the large number of clones from skin which secreted neither *IL-2* nor *IL-4*, but large amounts of tumor necrosis factor alpha *(TNFα)* and *IFNγ*.

In another study, following the analysis of cytokine m*RNA* expression by *RT-PCR* and cytokine secretion in in vitro stimulated peripheral blood mononuclear cells *(PBMC)* of leprosy patients, it was found that approximately *50%* of all individuals showed a *Th0*-like mixed cytokine pattern, irrespective of the clinical status of leprosy or the antigen used *(8)*.

Additional data from many laboratories have made important and also controversial contributions: i*)* addition of *IL-2* to patient cultures restored the *M.* leprae unresponsiveness in some lepromatous patients *(9);* ii*)* addition of *IL-4* to the cultures increased the pre-existing *M.* leprae response; iii*)* preincubation of patient cultures without any stimulus reversed the specific *T* cell unresponsiveness of lepromatous patients *(10);* iv*)* unresponsiveness to *M.* leprae could also be overcome in vitro by stimulation with *M.* leprae components in some patients.

In vivo immune reactivation in leprosy has also been demonstrated, as follows: i*)* changes in *T* cell populations were produced following *PPD*, r*IFNγ* and r*IL-2* injection, thereby eliciting upgrading aspects in the lepromatous lesions with increasing numbers of *CD4+* cells *(11-13);* ii*)* upgrading of lepromatous patients submitted to repeated vaccination *(BCG, M.* leprae, *M*ycobacterium *w) (14);* iii*)* presence of *M.* leprae-reactive *T* cells in lepromatous patients during the reactional episodes *(15,16);* iv*)* parameters of *T* cell activation have been observed in biopsies obtained from leproma-

tous patients during reaction; v) a marked influence on the immunological response to *M.* leprae (a positive response) was observed in some lepromatous leprosy patients submitted to specific chemotherapy *(17)*.

Immunological reactivity and cytokine production during reactions in leprosy

During the course of leprosy, a reasonable proportion of patients will develop reactional episodes of acute inflammation affecting the skin and nerves. Leprosy reactions provide a window through which one can observe the dynamics of the immune response to *M.* leprae.

These reactional states are classified as type *I (RR)* or type *II (ENL)* reaction depending on the clinical characteristics of the acute episode and its immune background. Both the precipitating factors and the physiopathological mechanisms involved in reactions remain ill defined. Although the outcome of this reactivation seems to be the elimination of bacilli or their constituents, irreversible peripheral nerve damage may occur in a small number of patients. The ultimate concern in leprosy must be the identification of inflammatory cytokines involved in the induction of tissue damage. Evidence implicating *TNF*α as a pivotal molecule in this process exists. *TNF*α seems to be a natural anti-infectious agent involved in bacterial resistance, which circumstantially provokes side effects at the site where elevated concentration is reached.

The acute episodes of immunological and inflammatory responses are associated with changes in immunological reactivity which seem to occur in both types of reaction. Tissue damage that occurs during the reactional states could be the result of opportunities generated during the evolution of *M.* leprae infection involving specific and non-specific cell-mediated immunity. The fragile equilibrium between activation and inhibitory molecules in the circulation and binding tissue molecules at the site of the skin infection is interrupted, generating a new order between pathogen and host. *TNF*α, a critical molecule related to tissue damage in leprosy, is overproduced during reaction *(18)*, and *IL-6*, *IL-8* and *IFN*γ may be associated secreted factors *(19)*. On the other hand, adhesion molecules constitutively expressed in the tissue or induced during the activation of cell-mediated immune response seem to collaborate, making the infected tissue more susceptible to the toxic effects of *TNF*α. The prompt beneficial effects obtained following treatment of reactional patients with thalidomide and/or steroids indicate that these episodes are not due to the aggravation of the infection itself, but are rather the result of the generation and release of excessively high amounts of host immunological mediators.

Histologically, reactional lesions contain a dense cellular infiltrate composed predominantly of lymphocytes *(CD4+T* cells*)*, monocytes and neutrophils *(*only seen in type *II* reaction*)*. This infiltrate is also accompanied by increases in epidermal thickness, keratinocyte *MHC*, class *II* antigen expression, an increased number of epidermal Langerhans' cells and the presence of intraepithelial *T* cells in the reactional lesion. Enhanced expression of *ICAM-1* on the keratinocytes and leukocyte function antigen *1 (LFA-1)* has recently been described *(19)*.

One may speculate that reaction occurs in the course of leprosy when a patient develops an increased cell-mediated immune response against *M.* leprae and, in some situations, the patient's clinical state could move toward the tuberculoid end of the spectrum.

Although very important conclusions can be drawn from a vertical study, only a sequential analysis of the immunological parameters from each individual patient will allow us to understand the actual mechanisms underlying the development of reactions in leprosy.

In an attempt to overcome part of these problems, we designed a longitudinal prospective study which would allow us to perform a sequential analysis in some patients. Accordingly, we started by addressing the following questions: 1) Could cell-mediated immunity arise from a previously unresponsive multibacillary leprosy patient during the reactional episode? 2) Would this T cell-mediated immune response be long lasting, moving the patient toward the tuberculoid end of the spectrum? 3) Would the cytokine profile determine these changes in the immune response? 4) Which cell populations participate in the triggering and regulation of these events?

Patients who developed a reaction during multiple drug treatment were submitted to immunological evaluation before, during and after the reactional episode and their data were described thereafter. Blood and tissue samples were collected from all patients during the study. Peripheral mononuclear blood cells were obtained by Ficoll-Hypaque density centrifugation. The lymphoproliferation assay, measurement of IFNγ in a 5-day culture supernatant and analysis of cytokine mRNA expression were performed in all patients. Histological and immunohistological analysis was done in 23 patients (a full description is given below). Serum samples were also collected from all patients before, during and after the reaction.

In this preliminary analysis, 66.6% of the patients tested showed an in vitro response to M. leprae during the reactional episode, as analyzed in the lymphoproliferation and IFNγ assays. When the immunological proliferative response to M. leprae was tested before and during the reactional episode, multibacillary patients who were unresponsive to M. leprae antigen became good responders during the reaction. More interestingly, the majority of patients who showed this positive immune response during the reaction shifted back to low responder status after the reactional episode subsided. Another important observation refers to the patient's response to the lepromin skin test. Although most patients became responsive to the skin test during the reaction, about 47.6% of them still had a positive lepromin response after the reactional episode.

Biopsies from 23 patients (a total of 48 samples) were submitted to immunohistochemical staining with a large panel of monoclonal antibodies. The number of γδ-TCR-positive cells as well as CD1-positive cells in the dermis was considerably increased during the reactions and remained relatively high in the post-reactional lesions. Gamma delta-T cells were not detected in pre-reactional lesions as opposed to the reactional lesions, which reached very high numbers in some cases. The CD4/CD8 ratio, although slightly >1 in pre-reactional lesions (mean 1.23 ± 0.36), was elevated in reactional lesions (1.77 ± 1.41) and remained high in post-reactional lesions (1.97 ± 1.56) as well.

There was an increased influx of CD4+ T cells to the site of reaction. The functional characterization of this T cell subset in each of the reactional states may elucidate some current questions. There is indirect evidence that the CD4+ Th1 subset predominates in these lesions since other parameters of local immune reactivation known to be induced by IFNγ are present in situ, such as increased expression of HLA-DR as well as ICAM-1 by keratinocytes and endothelial cells. Interestingly, these alterations remained high in post-reactional biopsies as well. The effect of IFNγ and TNFα in inducing keratinocyte membrane activation is very well known and may justify the changes detected in the reactional lesions. It is now widely accepted that overexpression of integrins on the endothelial cell surface is one of the first steps in the immuno-inflammatory cascade. These data indicate that the cell-mediated immune response is present in multibacillary patients during the reaction. The positive response of patients seen in vitro in the lymphoproliferation test and in the IFNγ assay supports this

conclusion. Another valuable contribution is the demonstration of the decay of cell-mediated immunity in the same patients tested at various times after reaction. By this time, lymphoproliferation (LTT) and IFNγ had returned to initial levels.

It seems that reversal reaction episodes occurring in unresponsive multibacillary patients may represent a unique moment in the course of leprosy infection, in which a break of the anergic state takes place.

A recent report (20) has demonstrated that the clinical course of a lepromatous leprosy patient was determined by the cytokine profile produced by different subtypes of CD4+ T cells. The predominant Th2 profile seen by RT-PCR during the disease was replaced by the Th1 profile on the occasion of reaction. This might indicate that studies on the profiles of cytokines in leprosy may not be reliable if done at only one time during the evolution of the disease. The decision as to which cytokine profile will be synthesized may lie in the balance between genetic factors and local environmentally acquired factors (IL-2, IFNγ, adjuvant, LPS, drugs, superantigens, vaccination, associated infections and others).

A sequential analysis of cytokine mRNA expression in the blood and in the lesions of patients from our cohort was of interest. Cytokine gene expression in PBMC was assayed directly after isolation from the blood and in the absence of any in vitro stimulation. Skin biopsies were also collected and the tissue was homogenized in a Polytron PT-3000 apparatus using Trizol (or RNAzol). Reagent RNA was purified according to manufacturer instructions and was then quantitated by absorbance readings at 260 nm. The integrity of RNA samples was monitored by agarose gel electrophoresis. Good quality RNA was obtained and was available for analysis by RT-PCR. After reverse transcription, cDNA was amplified for PCR evaluation in the presence of specific primer pairs for each of the cytokines tested (21).

For semiquantitative RT-PCR, PCR products were electrophoresed, transferred to nylon membranes and hybridized to the oligonucleotide probe. After successive washings, the filter was exposed to X-ray film for 24-48 h. Cytokine PCR products were quantitated using an analytic imaging system. The same amount of cDNA was used in each experiment. The results were normalized to the amount of actin in each sample, so that we were able to compare distinct intensity of the cytokine bands obtained from the lesions of different samples.

IL-1ß, IL-6, IL-8, TNFα, IFNγ, GM-CSF, IL-2Rp55 and perforin gene expression was detected mainly in reactional patients as compared to the other groups. Most of the unreactional patients were negative for the cytokines tested. None of the cytokine genes were detected in normal individuals or in lepromatous patients at the end of multiple drug treatment. In these patients, only actin mRNA was detected.

In the present study, it was possible to detect differences in TNFα gene expression in a lesion from the same patient when analyzed before and at the onset of a reactional episode. For the 8 patients tested, the amount of TNFα mRNA was increased in tissue during reaction, and was decreased thereafter following treatment. The expression of IFNγ mRNA was detected in the lesion of 4 out of the 6 ENL patients tested, whereas 3 out of 3 RR patients analyzed were positive for IFNγ in situ. Likewise, IFNγ gene expression in the blood and in tissue was down-regulated following treatment for reaction.

In summary, tissue studies also demonstrated that cytokines play a key role in all types of reactional lesions. Studies carried out on tissue lesions confirmed that TNFα-positive cells (as detected by immunocytochemistry) are a constant finding in reactional biopsies, although some positive cells can also be detected in non-reactional lesions. Moreover, using semiquantitative RT-PCR analysis, it was possible to detect dif-

ferences in *TNF*α gene expression in the lesion from the same patient when analyzed before and at the onset of a reactional episode. Thus, as demonstrated by kinetic studies, *TNF*α is undoubtedly increased during reactions in the same individual both in vitro (following stimulation of the cells with *TNF*α agonists) and in vivo (increased protein levels in the serum, and increased cytokine gene expression in situ).

Significant data suggesting that *IFN*γ molecules are in some way related to the triggering of *TNF*α overproduction were also obtained. The importance of *IFN*γ in the regulation of cytokine production was also suggested by the appearance of reaction in patients after intradermal injections of *IFN*γ (22). We have already demonstrated *IFN*γ priming of blood monocytes for enhanced agonist-induced *TNF*α release both in vitro and in vivo. Thus, *IFN*γ in the sera of leprosy patients could lead to priming of patient monocytes resulting in enhanced *TNF*α production and induction of reaction.

Within this context, the observation that *PBMC* obtained from reactional patients released higher *TNF*α levels than did monocytes obtained from the same individuals was intriguing. Interestingly, if purified monocytes are reconstituted in vitro with lymphocytes, levels of *TNF*α are similar to those released in the original *PBMC* cultures from the same individuals. It has recently been pointed out that *IFN*γ might be involved in *TNF*α overproduction. However, definitive studies showing that *IFN*γ is being synthesized in these cultures and its cell source are still missing.

In the present study, we were able to suggest that *1)* the borderline forms of leprosy are in principle clinically and immunologically unstable forms in which reactivation states are most likely to occur. *2)* The cytokine profile expressed by these patients either in vivo or in vitro may not be related to any pattern of cytokine secretion already described. *3)* The acute reactional episodes that develop in leprosy are associated with changes in cellular immune response since parameters of immune reactivation are detected both in vivo and in vitro. *4)* The immunological reactivation states seen along the chronic course of leprosy are the result of imbalanced local production and release of immuno-inflammatory cytokines (mainly *IFN*γ and *TNF*α) that may induce tissue injury.

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