Annex 5

**WORKING PAPER:**
Leprosy – new opportunities in basic science
LEPROSY – NEW OPPORTUNITIES IN BASIC SCIENCE

Douglas B Young
Centre for Molecular Microbiology and Infection, Imperial College, London SW7 2AZ, United Kingdom

The decade between initiation of the Mycobacterium leprae genome project and its final publication (1) witnessed a progressive decline in interest in leprosy research. This was caused by the perception that multidrug therapy provided a sufficient solution to the clinical problems of leprosy, the fact that Mycobacterium tuberculosis presented a more tractable experimental system than M. leprae for exploitation of the emerging techniques of mycobacterial genetics (2), and disappointing results with the heat-killed M. leprae vaccine developed under the IMMLEP programme (3). To take advantage of new opportunities arising from the genome sequence, there is a need to rebuild momentum in the leprosy research community. This can be achieved within a framework that combines research on the fundamental biology of leprosy alongside research that addresses practical aspects of leprosy control.

FUNDAMENTAL BIOLOGY OF LEPROSY

Study of the genetic make-up of M. leprae is an important component of the general investigation of evolution of human pathogens and is of particular relevance in the context of comparison with the closely related M. tuberculosis. Investigation of the dramatic neurological and immunological pathologies of leprosy provides a unique perspective that may increase understanding of normal human physiology.

Evolution of microbial genomes

The explosion of information from microbial genome sequencing projects has had a major impact in the field of evolutionary biology (4). There is an active interest in trying to understand how genetic exchange and genome reorganization have contributed to the evolution of different pathogenic strategies (5). M. leprae provides an interesting example as an evolutionary snapshot of a genome in transition (1). By comparison with M. tuberculosis, a quarter of the genome has already been deleted, and the large number of pseudogenes are thought to represent intermediates on the way to gene loss. M. leprae has undergone a process of adenine thymine enrichment that has been observed in several obligate pathogens (6). This may reflect loss of DNA repair enzymes that are required for correction of spontaneous cytosine to uracil deamination.

To pursue research in this area, there is a need to collect information about genetic variation amongst M. leprae isolates; experience with M. tuberculosis indicates that deletions and single nucleotide polymorphisms are likely to be particularly informative as evolutionary markers (7). Strategies to approach this would include detailed analysis of chromosomal DNA from one or more additional isolates prepared by armadillo passage. Partial coverage by shotgun sequencing would be sufficient to identify candidate polymorphisms. This could be complemented by PCR-based analysis of target loci in clinical samples to determine the extent of strain diversity at a population level. In addition to fundamental information about pathogenic mechanisms, investigation of M. leprae in an evolutionary context may provide some indication of when it adapted to human pathogenesis (8), and may allow us to determine whether it is now on an inevitable pathway to extinction. An interesting aspect of such studies involves examination of M. leprae DNA preserved in archaeological samples (9). This allows analysis of long-term changes in population structure, and could address the question of whether present day isolates of M. leprae differ from those that were prevalent in medieval Europe.

Host-pathogen interactions

Cellular microbiology – combining the tools of cell biology and microbiology – is at the forefront of current efforts to explore the host-pathogen interactions (10). In addition to its direct application to understanding pathogenesis, this field has generated useful insights into the fundamental biology of signal transduction and cytoskeletal organization in mammalian cells. The neural predilection of M. leprae offers an opportunity for a novel perspective on the organization and physiology of human peripheral nerves, and this has been exploited in a recent series of papers implicating phenolic glycolipid in neurotropism (11). There is considerable scope to extend these studies to investigate intracellular compartmentalization of M. leprae in Schwann cells and other cell types. It would be interesting to determine why M. leprae is found in multiple cell types during infection, for example, in contrast to the apparently restricted cell tropism of M. tuberculosis.

A central component of research on host-pathogen interactions involves analysis of changes in pathogen gene expression associated with adaptation to
an appropriate in vivo phenotype. The technical problems associated with direct analysis of in vivo phenotypes have been a central feature of leprosy research. Experience in isolating M. leprae from infected tissues and analysis of protein and lipid profiles may provide a useful model for parallel studies with other bacterial pathogens. Reciprocally, increased interest in the application of molecular approaches to histopathology may provide ways of examining leprosy lesions. Microarray-based whole genome expression profiling can be applied to characterization of the M. leprae transcriptome. This will depend on development of techniques for amplification of mycobacterial mRNA (12).

**Immunology**

There is considerable current interest in analysis of initial recognition of pathogens by innate immune mechanisms, and translation into signals that alert and direct the adaptive response. This is mediated in part by the family of toll-like receptors on macrophages and dendritic cells, and includes release of a series of proinflammatory cytokines (13). M. leprae can accumulate in tissues to much higher levels than M. tuberculosis, and appears to present a less potent proinflammatory stimulus. Comparison of responses to the two pathogens, together with knowledge of surface components, may be useful in identifying molecular determinants regulating innate immune recognition. Understanding of inflammatory responses to M. leprae is of particular importance in the context of leprosy reactions.

The specific absence of a Th1 response to M. leprae antigens in lepromatous leprosy represents a remarkable model for studying immune regulation. Evidence from analysis of leprosy lesions suggests that this may in part reflect polarization of the immune response towards a Th2 phenotype (14), but other mechanisms of tolerance remain to be investigated. It is not clear whether Th1 energy is a predisposing factor for lepromatous disease, or is a subsequent consequence of prolonged exposure to antigen. There is a current renewal of interest amongst basic immunologists in understanding mechanisms of peripheral tolerance and the role of regulatory T cell subsets (15); lepromatous leprosy would seem to provide an interesting challenge in this context. Findings from leprosy can be expected to generate insights into the less polarized immune changes underlying reactivation tuberculosis. While the immune response in infected humans should be the main target for research, experiments in genetically manipulated mouse strains may assist in dissecting fundamental aspects of immunity to M. leprae (16).

**Host genetics**

Twin studies demonstrate that there is an important genetic element in susceptibility to mycobacterial disease (17). Considerable effort has been invested in searching for genetic determinants of susceptibility to tuberculosis in humans and in animal models. Few clearcut results have been obtained; it is likely that multiple loci contribute to susceptibility. For leprosy, the presence of well-established clinical phenotypes may offer an advantage in facilitating selection of tightly defined cohorts for genetic screens (18,19).

**SUPPORTING LEPROSY CONTROL**

In spite of the success of multidrug therapy over the last decade, there has been no obvious decline in the number of new cases of leprosy reported from high incidence countries (20). This raises the question as to whether passive case finding and treatment is sufficient to interrupt leprosy transmission, or whether additional intervention tools are required. The M. leprae genome offers a range of research opportunities of direct relevance to current control strategies.

**Drugs and drug resistance**

The emergence of multidrug resistant organisms has had a major impact on tuberculosis control. Although dapsone resistance was widespread in the pre-MDT era, rifampicin resistance has not yet presented a major problem for leprosy control. Molecular genetic tests for mutations associated with rifampicin resistance provide a convenient surveillance tool to monitor this situation (21). From a similar perspective, it would be of interest to identify the genetic basis of M. leprae resistance to other drugs.

While it is unlikely that novel drugs will be developed specifically for treatment of leprosy, it is important to have the ability to test whether newly developed antibiotics have any potential application in improved leprosy treatment. Assays based on measurement of transcriptional activity, and perhaps the use of reporter phage constructs (22), provide possible avenues for exploitation of genetic tools in development of novel tests to assess the effects of drugs on M. leprae.

**Transmission**

Monitoring transmission of M. leprae may provide a powerful tool for assessment of the impact of leprosy control strategies, since reduced transmission may precede a reduction in disease incidence by years or even decades. In principle, transmis-
Mycobacterial infection can be detected by antigen-specific immune responses manifest by delayed type hypersensitivity, T cell proliferation, or cytokine release. Antibody production represents an alternative readout, though this is likely to require presence of a higher concentration of antigen and may therefore be less sensitive. The most convenient current technologies involve measurement of interferon-\( \gamma \) production by exposure of peripheral blood T cells to synthetic peptide antigens. The readout involves detection of soluble cytokine by enzyme linked immunosorbent assay (ELISA), or enumeration of cytokine-producing T cells by ELISpot. In the tuberculosis field, considerable enthusiasm has been generated by an ELISpot assay based on the ESAT6 antigen \( (23) \). Comparative genomics offers a powerful solution to the prolonged search for \( M. \) leprae-specific antigens for use in such an assay. Simple bioinformatic tools can be used to screen the limited panel of \( M. \) leprae-specific open reading frames to identify peptides that include appropriate major histocompatibility complex binding motifs and lack cross-reactive homologues \( (24) \). Development of a specific test for \( M. \) leprae infection represents a very feasible short-term goal.

Molecular epidemiology has taken on an important role in tuberculosis research, with the use of strain typing to confirm reactivation disease \( (25) \), to distinguish reinfection from relapse \( (26) \), and to estimate the prevalence of disease due to recent transmission \( (27) \). When disease arises predominantly as a consequence of recent transmission, the resulting isolates tend to share the same genetic features. When disease results from reactivation of some earlier infection, isolates are more likely to be genetically diverse. It is anticipated that the molecular epidemiology of leprosy will follow a similar pattern and that strain typing would therefore help clarify the relative importance of recent transmission within an endemic community. Several polymorphisms have been described for strain typing of \( M. \) leprae \( (28-30) \). The number of copies of a non-coding triplet repeat sequence shows considerable variation between clinical isolates \( (30) \). A diverse range of triplet repeat patterns was found in a panel of isolates obtained from the same single leprosy clinic at Hyderabad in India (SK Young, GM Taylor, unpublished), suggesting that this polymorphism may be useful for localized epidemiological mapping. Tools for the molecular epidemiology of leprosy will be generated by the fundamental research on evolutionary biology of \( M. \) leprae described above, and will open up exciting new opportunities for practical application.

### Nerve damage

The need for new tools to assist in prevention of nerve damage during leprosy treatment has consistently been viewed as a high priority in leprosy research. Basic research on neurotropism and on immunology clearly have potential relevance in this area, which is discussed in detail in an accompanying working paper.

### Vaccines

The search for an effective prophylactic vaccine provided a central theme for TDR-sponsored leprosy research in the 1980s. An important finding from the resulting clinical trials was that BCG consistently confers protection against leprosy, and that this protection is boosted by a second BCG vaccination \( (31) \). Both of these findings appear to be in contrast to experiences with the use of BCG against tuberculosis, and the repeat BCG result tends to contradict the prevailing thought that BCG is ineffective in individuals with pre-existing mycobacterial immunity \( (32-33) \). A second mycobacterium, the ICRC bacillus, was also shown to elicit a protective effect, in this case after delivery as a killed vaccine \( (3) \). In terms of leprosy control, it would seem attractive to include repeat BCG (or an alternative vaccine) as part of any targeted strategy to reduce leprosy in high incidence areas. From a research perspective, it would be interesting to try and understand the nature of the immune response that is boosted by repeat BCG and that confers protection against \( M. \) leprae.

### SUMMARY

Leprosy offers an interlinked series of research topics ranging from fundamental biology to practical application. To exploit these opportunities, there is a need to bring about some restoration of the extent and momentum of the leprosy research community. TDR can play a role in this by publicizing research opportunities, coordinating meetings of basic and applied scientists working on leprosy and related topics, and supplying seed money to encourage new investigators to take an interest in leprosy.

### References


Annex 6

WORKING PAPER: The impact of multidrug therapy on trends in transmission
THE IMPACT OF MULTIDRUG THERAPY ON TRENDS IN TRANSMISSION

Abraham Meima
Erasmus University Rotterdam, Department of Public Health Medical Decision Sciences, Rotterdam, The Netherlands.

LITERATURE REVIEW OF TRENDS IN LEPROSY NEW CASE DETECTION RATES

As a first step in investigating the impact of case detection and multidrug treatment (MDT) on leprosy, a literature review was conducted of trends in leprosy new case detection rates (NCDRs) as published in international literature (1). This review covered nine countries and seven smaller geographical entities, and NCDR data up to 1993. For the majority of the areas/countries, NCDR trends were declining (13 of 16 areas/countries had an average annual decline in NCDR of at least 2% per year). The literature review may have suffered from publication bias, i.e. a tendency to publish papers on high quality control programmes covering long periods of time or indicating successful leprosy control. In the review, an acceleration of declines in trends after the introduction of MDT was not visible. The long incubation period of leprosy could have masked such accelerations. For seven of the nine countries, additional country data became available (2-7), which allowed for extension of the NCDR time series. A general impact of MDT on NCDR trends can still not be demonstrated.

NEW CASE DETECTION RATE TIME SERIES CONSTRUCTED ON THE BASIS OF COUNTRY DATA FOR 1985-2001

For 14 countries with at least 2000 newly detected cases in 1998, NCDR times series could be constructed from the year 1985 onwards using country data obtained from subsequent issues of the Weekly Epidemiological Record (3-7) and from one conference report (2). The NCDR trends are highly variable, and underlying trends in leprosy transmission are unclear. Most probably, operational factors heavily influenced the NCDR trends: leprosy control activities were intensified following the 1991 World Health Assembly resolution to “eliminate leprosy as a public health problem by the year 2000”, and leprosy elimination campaigns were initiated. For 7 of 14 countries, NCDRs in the 1990s were either rather stable, or increased (Bangladesh, Brazil, Ethiopia, India, Indonesia, Mozambique, Sudan). For 3 of 14 countries, sudden sharp increases in NCDRs were observed in the late 1990s (Myanmar, Nepal, Madagascar). For 3 of 14 countries, a decreasing tendency – at least in more recent years – was observed (Philippines, China, Vietnam). For the remaining country (Guinea), strong fluctuations in the NCDR were observed.

REASONS FOR DECLINING TRENDS IN THE TRANSMISSION AND INCIDENCE OF LEPROSY

Declines in transmission and incidence (i.e. onset of disease) of leprosy may be related to several factors:

- The period during which _M. leprae_ is transmitted, which can be reduced by early case detection and chemotherapy treatment.
- BCG vaccination, which is widely administered as a preventive measure against tuberculosis but appears to afford more protection against leprosy than tuberculosis (8).
- Socioeconomic conditions, which are thought to play an important role in leprosy (9). Their improvement may result in a decline in incidence. Factors suggested to contribute are housing conditions, number of persons per household per room, family size and nutritional factors.
- Possible protection of tuberculosis against leprosy (10), either by immunization or by competing risk.

FACTORS POSSIBLY LIMITING THE IMPACT OF BCG

In most developing countries, BCG is given in young childhood, and is only given once. Protective efficacies against leprosy ranged from 20% to 80% in randomized controlled trials (8). But the protection was quite small in Asia where most patients are detected (two trials in India: 24% and 34% (11); one trial in Burma: 20%). It is also unclear whether the protection decreases with age. Thus, the impact of BCG on leprosy trends may be smaller than one would hope for. The impact also depends on

---

6 Countries in the literature review: Philippines, Bhutan, Thailand, Malawi, Ethiopia, Rwanda, Brazil, Guyana, Mexico. Other areas: French Polynesia, Wenshan Prefecture (China), Weifang Prefecture (China), Visakhapatnam District (India), Shoa Region (Ethiopia), Uele Region (former Zaire), 3 Northern Provinces (Thailand).

7 The 14 countries are: Bangladesh, Brazil, China, Ethiopia, Guinea, India, Indonesia, Madagascar, Mozambique, Myanmar, Nepal, Philippines, Sudan, Vietnam.
A further problem is the delay between onset of disease and detection. For instance, in the ALERT control programme in Ethiopia, the average detection delay exceeded two years. How easily leprosy is transmitted is not known. The group at risk of developing leprosy might be small, possibly due to genetic factors (leprosy infection is suggested to be much more common than leprosy disease [10,12]) or because close contact is important. Close contact – household and family, neighbours, social and business contact – has been suggested to play a key role in transmission (13). It is well possible that close contacts of a leprosy patient become infected rapidly. If close contact is indeed important, this may lead to a rapid decrease in the patient’s opportunities to transmit M. leprae. Thus, “early” detection may still be too late to prevent much of transmission by subsequent treatment. Other factors which could limit the impact of leprosy control have also been suggested, including carriage of M. leprae in the nose, persistence of M. leprae in the soil, and even animal reservoirs (14-17).

LEPROSY SIMULATION MODEL

An epidemiological model, SIMLEP, was developed in order to assess the impact of interventions on transmission. In this model, populations are divided in mutually exclusive compartments, and a set of calculation rules is used to determine the number of transitions between the compartments in subsequent time steps (18). Through this approach, trends over time can be simulated in transmission and onset of disease, case detection, numbers of incubating individuals, undetected leprosy patients, and patients on treatment.

ANALYSIS OF DISAPPEARANCE OF LEPROSY FROM NORWAY USING SIMLEP

Leprosy was still an endemic disease in Norway around 1850, but had virtually disappeared by 1920, long before effective anti-leprosy treatment became available. The downward trend is extremely well documented (19). The decline coincided with continuous growth of the Norwegian economy. In Norway, a policy of isolation of patients was implemented. By legislation in 1877 and 1885, leprosy patients either had to be isolated in separate rooms in their houses, or had to be admitted to a hospital.

The downward trend of new case detection in Norway was adequately reproduced with SIMLEP, and equally well for 8 (2 × 4) pairs of assumptions on contagiousness during the incubation period and on decrease of transmission opportunities (build-up of contagiousness during incubation period: yes/no; half-value time for transmission opportunities: “none”, 2, 4 and 8 years). However, the estimated contribution of hospital isolation to the decline of leprosy in Norway ranged from only 3% to 60% for these 8 pairs of assumptions, the other explanation being the socioeconomic development. Thus, the impact of isolation proved to be very uncertain.

“NATURAL EXPERIMENT”

The Norway study showed that declining trends in leprosy transmission may have competing explanations. A logical next step in a model-based approach would be a SIMLEP-based analysis of long-term trends in (preferably adjacent) geographical areas with comparable general conditions, but with different well documented leprosy control policies. Such trend data would enable disentanglement of competing explanations (MDT, BCG, socioeconomic change) for decreases in transmission, but unfortunately they are not readily available. So far, we have not identified suitable datasets.

SCENARIO ANALYSIS OF LEPROSY TRENDS UP TO 2020 USING SIMLEP

The effect of MDT is similar to that of isolation: both prevent leprosy transmission. Trend predictions are complicated by the same factors that were encountered in the evaluation of the Norwegian decline. Building on the Norwegian experience, a scenario analysis of future trends in leprosy incidence was conducted with SIMLEP (20).

For each of the 8 pairs of assumptions on contagiousness during the incubation period and on

LAUNCH OF THE WORLD ACTION PLAN ON LEPROSY

The World Health Assembly (WHA) of May 2001 called for an action plan to reduce the number of new cases of leprosy by 80% by 2005 in comparison with the year 1995-1999 and to eliminate leprosy by 2050. This plan, the World Action Plan on Leprosy, was launched in 2001. It is being implemented in 120 countries and territories with a total of 1.2 billion people at risk of leprosy. The plan is supported by the Special Initiative for Leprosy, which is funded by the global community and coordinated by WHO. The plan is being implemented in collaboration with local partners in order to reach the most vulnerable groups, especially those who are not easily accessible to health care services. The plan is aimed at improving access to diagnosis and treatment, reducing the social and economic impact of leprosy, and helping countries to build sustainable leprosy control programmes.

This plan is being implemented in collaboration with local partners in order to reach the most vulnerable groups, especially those who are not easily accessible to health care services. The plan is aimed at improving access to diagnosis and treatment, reducing the social and economic impact of leprosy, and helping countries to build sustainable leprosy control programmes.

The World Action Plan on Leprosy is being monitored through periodic reports on the progress of the plan. These reports are published in the WHO Bulletin and provide an overview of the progress made towards the targets set by the WHA in 2001. The reports also highlight the challenges faced in implementing the plan and the lessons learned from successful interventions. The reports are used to adjust the plan as necessary and to ensure that the plan is on track to achieve its goals.

The World Action Plan on Leprosy is being monitored through periodic reports on the progress of the plan. These reports are published in the WHO Bulletin and provide an overview of the progress made towards the targets set by the WHA in 2001. The reports also highlight the challenges faced in implementing the plan and the lessons learned from successful interventions. The reports are used to adjust the plan as necessary and to ensure that the plan is on track to achieve its goals.

This plan is being implemented in collaboration with local partners in order to reach the most vulnerable groups, especially those who are not easily accessible to health care services. The plan is aimed at improving access to diagnosis and treatment, reducing the social and economic impact of leprosy, and helping countries to build sustainable leprosy control programmes.

The World Action Plan on Leprosy is being monitored through periodic reports on the progress of the plan. These reports are published in the WHO Bulletin and provide an overview of the progress made towards the targets set by the WHA in 2001. The reports also highlight the challenges faced in implementing the plan and the lessons learned from successful interventions. The reports are used to adjust the plan as necessary and to ensure that the plan is on track to achieve its goals.

This plan is being implemented in collaboration with local partners in order to reach the most vulnerable groups, especially those who are not easily accessible to health care services. The plan is aimed at improving access to diagnosis and treatment, reducing the social and economic impact of leprosy, and helping countries to build sustainable leprosy control programmes.
decrease of transmission opportunities, model projections were fitted to reference data on leprosy new case detection from 1985 onwards (the reference case detection rate was calculated as the average of the NCDR of the 14 countries that detected at least 2000 cases in 1998, and for which data are available at country level throughout 1985-1998). In doing so, it was assumed that the delay in case detection decreased in the 1990s, which is in line with the intensification of control efforts that took place in many countries. Subsequently, incidence rates were predicted up to the year 2020, assuming an average detection delay of 2 years from 2000 onwards. Again, wide variation was observed in the projections: the annual decline in incidence rate between 2000 and 2020 ranged from 2% to 8% per year for those pairs of assumptions for which a reasonable fit of the reference data was obtained. The corresponding times to reduce the incidence rate by 50% were 43 and 8 years. The rates of decline were lower with contagiousness during the incubation period, and when a faster decrease in transmission opportunities for patients was assumed.

So far, there was no BCG vaccination. The scenario analysis was repeated while making quite favourable assumptions on BCG: 50% lifelong protective efficacy, and rather optimistic coverages. Now, the annual decline in incidence rate ranged from 5% to 10% per year, with corresponding times to reduce the incidence rate by 50% of 14 and 7 years. Thus, leprosy incidence declined in all scenarios considered, and BCG enhanced the declines. The most important conclusion is the slow pace at which the incidence is reduced in all scenarios.

**CONCLUSIONS**

- MDT based control appears to reduce transmission. The pace of reduction is highly uncertain, but in any case slow. BCG may enhance the pace, but its impact is also uncertain.
- The impact of MDT based control is highly uncertain because of the following unknowns:
  - the role of close contact in transmission
  - the speed of transmission
  - whether, and to what extent, contagiousness builds up during the incubation period.
  Research addressing these questions is essential to narrow down the uncertainty regarding the impact of MDT based control.
- Further progress through SIMLEP requires data which allow for disentanglement of competing explanations (MDT, BCG, socioeconomic change) for downward trends in leprosy transmission. This requires the availability of long-term trend data from geographical areas with comparable general conditions, but with different well documented leprosy control policies.
References


Annex 7

WORKING PAPER: Current status in reactions and nerve damage in leprosy – what next?
CURRENT STATUS IN REACTIONS AND NERVE DAMAGE IN LEPROSY – WHAT NEXT?

Elizabeth P. Sampaio, Maria Cristina F. Pessolani, Milton O. Moraes, Euzenir N. Sarno
Leprosy Laboratory, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil.

PATHOGENESIS OF REACTIONS AND NERVE DAMAGE IN LEPROSY

Notwithstanding the application of the Th1xTh2 paradigm to the polar spectrum of leprosy (1), the occurrence of reactions provides new insights into the mechanisms of immunological regulation during host-pathogen interaction. Reactions might, in fact, represent an immune-inflammatory breakthrough of the steady-state present in a previously unresponsive (multibacillary [MB]) or low responsive (MB or paucibacillary [PB]) patient that can temporarily revert to a highly responsive immunological profile and inflammatory condition, at which time the patient’s clinical state could move toward the tuberculoid end of the spectrum. The potential for patients to develop any type of reaction depends on multifactorial variables that would necessarily include genetic features, histocompatibility leukocyte antigen (HLA) haplotype, immunological background, and bacillary load, among others. This is particularly true in light of the observation that erythema nodosum leprosum (ENL) is most often experienced by lepromatous patients (borderline lepromatous [BL]/lepromatous leprosy [LL] forms), reversal reaction (RR) most often develops in borderline borderline (BB) patients, while both types of reaction tend to occur at a similar frequency rate in BL patients (2).

It is widely accepted that up-regulation of the cell mediated immune (CMI) response, which is dependent on the expansion of M. leprae-specific T cells, occurs during RR (3). The re-emergence of CMI seems to be related to the breakage of the anergic state that possibly occurs in patients who present a low immune response level (borderline tuberculoid [BT], BB, and BL patients). RR is, consequently, associated to the up-regulation of IFNγ production leading to granuloma formation, enhanced macrophage microbicidal activity, and inflammation (4,5). RR patients are able to respond in vitro to M. leprae and in vivo to the lepromin skin test, and RR has been associated with enhanced bacterial clearance (systemic bacterial load) when compared to unrea-

Do reversal reactions and erythema nodosum leprosum share a common immunological background?

Accumulated evidence suggesting the participation of CMI in ENL includes:

- The positive lymphoproliferative response in conjunction with increased IFNγ production in response to M. leprae in vitro (7).
- The detection of interferon-gamma (IFNγ) and interleukin (IL)-12 mRNA expression in the lesions and blood of reactional patients (8,9).
- The finding that T cells isolated from the lesions and/or blood of leprosy patients have a Th0 or Th1 profile in vitro (10).
- The involvement of activated T cells in the enhanced tumour necrosis factor-alpha (TNFα) production induced in M. leprae-stimulated monocytes through direct cell-cell contact (11).

The occurrence of sequential ENL and RR in one individual patient may indicate further that both types of reaction share a similar immunological background. An identical mRNA cytokine profile was observed in the lesions of this same patient during the course of reaction, although gammadelta (γδ) T cells were only present in the RR and not in the ENL lesion (12). Despite the similar immunological profile detected in vivo, the histological and clinical features observed in both types of reaction are diverse. In fact, in MB leprosy (when rescue of specific CMI is more easily achieved, as in the borderline forms), immune reactivity is capable of emerging spontaneously (the so-called reactions) and, depending on the number of bacteria, HLA restriction, and spatial/temporal cell type activation, this immune reactivation is clinically observed as either RR or ENL.

It is worth considering that, due to the dynamic but rather chaotic regulation of cytokine secretion (13,14), even the most subtle activation process may be capable of triggering an exacerbated inflammatory response. In any case, detection of IFNγ in vitro in response to M. leprae has only been observed in approximately 30% of all ENL patients studied (Sampaio et al., 16th International Leprosy Congress), despite the fact that the majority of RR and ENL patients expressed IFNγ and IL-12 mRNA in the skin while undergoing reaction. The detection of such cytokines in the tissues of patients in the
absence of detectable IFN\(\gamma\) production in vitro raises at least two interesting hypotheses:

- It is possible that a transient IFN\(\gamma\) production by Th2 cells is operative in the lesions of ENL patients in contrast to what, in RR patients, appears to be a more long-term, established production of IFN\(\gamma\) (and TNF\(\alpha\)) by Th1 clones. In fact, it has been reported that, in addition to the IL-12-induced differential expression of the \(\beta2\) subunit of the IL-12 receptor (IL-12R\(\beta2\)) on established Th1 cells (15), an induced temporary up-regulation of the IL-12R\(\beta2\) transcripts in Th2 clones may account for the transient production of IFN\(\gamma\) in cells with an established Th2 phenotype (16). The quantification of IL-12R transcripts as well as of the subtle differences in mRNA expression (IFN\(\gamma\), TNF\(\alpha\), IL-12) of ENL vs. RR in situ clearly require further investigation.
- An alternative source of IFN\(\gamma\) may originate from the very early components of the innate immune response. Cells from the innate immunity (dendritic cells, monocytes, natural killer [NK] cells) shape the nature of the subsequent adaptive T cell response by influencing the cytokine pattern. The activation of NK cells (or \(\gamma\delta\) T cells) induced by \(M. leprae\) together with the production of IFN\(\gamma\) may occur early on following antigen-presenting cell (APC) stimulation with the concomitant secretion of IL-27, interferon \(\alpha\), and IL-12 (17). IL-12 is a dominant factor in driving the development of a Th1 response leading to IFN\(\gamma\) secretion on the part of NK and T cells. In this connection, it has been further demonstrated that IL-27 (an early product of pathogen- or lipopolysaccharide [LPS]-stimulated APC) is capable of eliciting a potent response from naive (but not memory) CD4+ T and NK cells, in conjunction with an early induction of IFN\(\gamma\) (18) that can also occur independently of IL-12. It is also suggested that IL-27, although it may play a role in the rapid initiation of a response to an inflammatory challenge, appears to be unnecessary for its maintenance. It is, therefore, plausible to postulate that, during ENL, IL-27 and/or IL-12 and IL-15 are involved in the early IFN\(\gamma\) release by NK cells (and naive T cells) that are ultimately responsible for directing the immune response not specifically related to the reactivation of memory T cells. Conversely, in RR, besides the aforementioned cytokines, IL-18, which appears to act at a later stage in Th1 development, will induce, in synergy with IL-12, further secretion of IFN\(\gamma\) by the differentiated T cells. IL-21 (19) and IL-23 (17,18) may then come into play in the process of transition to acquired immunity and on the selective activation of memory T cells, respectively (RR).

Hence, the precise role and expression of these newly described mediators in leprosy, and perhaps others still unknown, certainly merit investigative research.

**Major points for further investigation**

- Which cells are able to release IFN\(\gamma\) during the reactions? CD4, CD8 T cells, NK cells, \(\gamma\delta\) T cells?
- Kinetics of the cytokine response in RR vs. ENL.
- Role of the innate immune response in leprosy reactions.

**Are other mediators related to the enhanced secretion of TNF\(\alpha\) and induction of tissue damage during reactions?**

Leprosy reaction has been related to the overproduction of TNF\(\alpha\) both in vivo and in vitro. Our data and those of others (20-23) have suggested that TNF\(\alpha\) is a key mediator in the immunopathology of tissue damage in both RR and ENL. It has been hypothesized that the emergence of the innate or acquired immune response in ENL, followed by production of IFN\(\gamma\), IL-6 and IL-12 in situ, could be synergistically involved in the amplification of the inflammatory response and in the augmented release of TNF\(\alpha\), both locally and systemically (8,24,25).

In our own study, IFN\(\gamma\) was demonstrated to enhance the \(M. leprae\)-induced secretion of TNF\(\alpha\) and the rate of macrophage apoptosis both in vivo and in vitro (21, Hernandez et al., submitted for publication). On the other hand, it is also possible to postulate that activation of monocytes by LPS-related molecules, through the CD14 receptor and/or the Toll like family of receptors (TLR) required for optimal induction of innate immunity, might be operative. In that sense, the expression and secretion of inflammatory mediators will be preferentially detected in ENL independently of IFN\(\gamma\), and will mediate the systemic damaging effects observed during the reaction. Both TNF\(\alpha\) and TLR have been implicated in triggering cell death in response to mycobacterial infection, which might be related to bacterial killing as well. Accordingly, \(M. leprae\) (similarly to \(M. tuberculosis\)) has recently been shown to induce apoptosis in monocytes (Hernandez et al., submitted for publication) and to activate NF-kappaB transcription factor (Hernandez et al., 16th International Leprosy Congress). In the same study, thalidomide and pentoxifylline, well known TNF\(\alpha\) inhibitors, were able to decrease apoptosis in paral-
el to the diminished TNFα release (26). Moreover, the 19 kDa of M. leprae seems to activate the TLR2 and to induce apoptosis in vitro (Oliveira et al., submitted for publication).

The balance between pro- and anti-inflammatory cytokines induced in response to bacterial products in vivo seems to be related to the induction and resolution of inflammation in several diseases as well as in leprosy. Follow-up evaluation of cytokine mRNA expression in reactional skin in the dermis (24,25) and epidermis (27) indicated that improvement of patients’ clinical symptoms following in vivo administration of anti-inflammatory treatment (prednisone, thalidomide, or pentoxifylline) was associated with decreased expression of TNFα, IFNγ, and IL-12 (24,25) mRNAs; on the other hand, worsening of their clinical conditions was correlated to the maintenance/induction of cytokine mRNAs levels, including IL-10 and IL-4. These cytokines might be a contributing factor in the deleterious effects classically attributed to TNFα. The notion that their overwhelming or unbalanced production can contribute to undesirable inflammatory side-effects must be validated. Several data point to IL-12 as playing a critical role in the pathogenesis of Th1-mediated autoimmune diseases (28). Monitoring of cytokine mRNA expression in situ can allow early indication of occurrence and evolution of reactional inflammation in leprosy.

Studies on the kinetics of cytokine production in vitro have revealed that IL-10 is produced later in culture and down-regulates the production of cytokines synthesized earlier, as well as its own production. In addition, IL-10 secretion by monocytes has been shown to be regulated by TNFα. Enhanced TNFα secretion seems to play a key role in the sustained inflammatory manifestations during leprosy reactions, and it is postulated that a different profile in the kinetics of TNFα and IL-10 released in response to M. leprae might be detected in vitro in reactional versus unreactional patients. Kinetic studies showed early and enhanced M. leprae-induced TNFα production (mRNA and protein) from ENL patients’ cell cultures followed by a delayed IL-10 response (Sampaio et al., in preparation for publication). Hence, the data favour the idea that, in a low responsive patient, IFNγ can overcome patient’s unresponsiveness by inhibiting IL-10 and augmenting IL-12 production.

In addition to TNFα, several other mediators are most likely involved in the induction of tissue damage. The remodelling of extracellular matrix components requires the action of proteases, among which are matrix metalloproteinases (MMPs), zinc-binding proteins that appear to be stimulated by cytokines such as IL-1, TNFα, and phorbol esters. MMPs and their inhibitors also play an important role in the tissue remodelling which accompanies inflammation, wound healing, tumour invasion, and bone resorption, and have been implicated as potential contributory factors in the pathogenesis of inflammatory skin disorders. In leprosy, enhanced mRNA expression of both MMP-2 and MMP-9 (gelatinase proteins) has recently been detected in reactional but not unreactional leprosy tissue (Teles et al., in preparation for publication). Additionally, cytotoxic genes have been implicated in such typical immunopathological events as transplant rejection. Although our preliminary analysis in peripheral blood mononuclear cells (PBMC) showed no major differences in mRNA expression of granulysin, Fas L, and granzyme B among the patients, investigation of perforin mRNA carried out in biopsies showed expression of message in 50% of RR and 100% of ENL, but not in unreactional patients. The data suggest that cytotoxic T cells are being recruited to the lesion site during the reactional episode and that they (and the MMPs) may well be participating in generation of the tissue damage detected during the reactions.

Although HLA alleles may play a role in controlling development of the type of leprosy, genetic predisposition is only one factor in the complex process leading to disease, the end result of which is probably dependent on the interplay of several host genes. A variety of other immunogenetic polymorphisms seems to be implicated in human infectious diseases, cytokine genes being the more relevant ones. Notwithstanding the overlapping mechanisms involved in the immune regulation during reaction, it is known that genetic background influences, for example, approximately 60% and 75% of TNFα and IL-10 production, respectively. Polymorphisms in a number of genes (such as IFNγ, IL-10, IL-4, TNFα) and molecules (such as natural resistance associated macrophage protein 1 [NRAMP1], toll-like receptor 2, vitamin D receptor) have been described as being associated with protection and susceptibility, and with severity, in leprosy. Such genetic patterns may well explain the differences found in the profiles of response observed in lepromatous vs. tuberculoid patients. Single nucleotide polymorphism (SNP) at position -308 (guanine/adenine) within the promoter region of the TNFα gene was associated with the development of lepromatous leprosy in India (29). However, in the Brazilian population, it was demonstrated that controls showed higher frequencies of the -308A TNF mutation as compared to leprosy patients as a whole or MB patients (30). In a subsequent study, a stronger lepromin skin test reaction was detected in the PB patient population.
(-308A carriers) when compared to the non-carriers (31); even so, among those -308A TNF carriers who experienced continuous skin inflammation (leprosy reactions and BCG vaccination), a decreased skin test response was evidenced. The data suggest that TNFα promoter SNPs may influence disease susceptibility and also indicate that a genetic background favouring high levels of TNFα production in vivo may trigger an immunomodulatory after-effect to control the exaggerated response. A role for TNFα in protection in humans was confirmed when the occurrence of tuberculosis (32) (and leprosy [Joyce et al., 16th International Leprosy Congress]) was detected in patients following in vivo inhibition of TNFα production. In patients with reaction, TNFα promoter SNPs could then indicate high or low TNFα production and could be used as prognostic markers in preventing strong inflammatory reaction and excessive tissue damage. On the other hand, enhanced levels of TNFα have been noted in the serum of both -308A and -308G carriers in the course of leprosy reaction (33), suggesting that other factors may also be contributing to TNFα hyperresponsiveness. As related to IL-10, it has also been described that the IL-10 haplotypes located in the distal promoter region of the gene appear to be associated to disease severity in leprosy (Moraes et al., 16th International Leprosy Congress). Altogether, the results indicate that specific cytokine SNPs could be used as susceptibility and severity markers by genetically screening the high risk population among both household contacts and patients, thereby providing a novel tool for predicting susceptibility to disease and/or later complications, such as nerve injury.

**Major points for further investigation**

- Is TNFα being induced in ENL through an IFNγ independent manner? Toll like receptor? CD14 molecule?
- Definition of risk factors and/or markers for development of reactions.
- Application of anti-TNFα therapy to modulate the clinical manifestations of reactions/prophylactic treatment for reactions (Zafirlukast, infliximab, anti-TNFα antibodies, anti-TNFR antibodies, azathypoprine, cyclosporine, thalidomide analogues).

**NERVE DAMAGE IN LEPROSY**

At this particular moment, it is generally agreed that the most fascinating field of leprosy research lies in elucidating the peculiar context in which this particular bacterial infection terminates in such specific nerve destruction. Nerve damage occurs across the entire spectrum of leprosy but is more often seen at the lepromatous pole, especially among borderline patients, who can present the most extensive nerve impairment. In some cases, irreversible nerve injury progresses insidiously and largely unnoticed for long periods of time, in marked contrast to the acute and/or subacute injury of peripheral nerves that occurs in the course of the reactional states seen in patients undergoing chemotherapy. A finding of considerable clinical importance is that many patients, even after terminating MDT and therefore lacking viable M. leprae, nevertheless present significant nerve damage and persistent neuritis. Whether this is due to dead bacteria or the attempt to clear the bacteria or their components is not clear. Steroid therapy administered prior to the onset of significant nerve damage is often able to prevent these permanent disabling symptoms. However, in some cases, prompt and adequate oral prednisone seems to have very little effect on the recovery of persistent nerve lesions (34).

Several pathogenic mechanisms, including biochemical interference of the bacillus with host cell metabolism, mechanical damage due to the large influx of cells and fluid, and/or immunological damage, may be responsible for nerve damage in leprosy. It is known that successful axonal regeneration occurs exclusively within the endoneurial tubes lined by Schwann cell (SC) basal laminae, and that degradation and remodelling of extracellular matrices are important aspects of the regenerative process (35). Antigen-specific T cell-mediated hypersensitivity to M. leprae antigens, which is more frequently observed during acute reversal reactions, has been associated with the pathogenesis of nerve lesions in the tuberculoid forms of leprosy. However, the mechanism of nerve damage in the lepromatous forms is still uncertain. Because reactions are accompanied by an increased CMI response, and because acute nerve lesions particularly occur during these episodes, defining the role of the immuno-inflammatory response deserves particular consideration (36,37). Taking into account that the neural lesion is specifically related to M. leprae infection, the release of some mediators induced by this pathogen would probably account for nerve destruction, either directly or indirectly. Based on the available literature implicating TNFα in the pathological mechanisms of several nervous system diseases, this cytokine has been detected in the sera of both MB (ENL and RR patients) and PB patients with neuritis although, as expected, at lower levels than in ENL (36). Therefore, TNFα has been considered one of the possible prime movers in mediating nerve injury.
How is it that TNFα released within the nerve environment is able to destroy the nerve structure in such a way as to obliterate its regenerative capabilities?

The expression of TNFα mRNA and protein in the skin and nerves of RR patients (23) points to TNFα as participating in the pathophysiology of nerve damage in leprosy. Moreover, there is mounting evidence that TNFα contributes to the pathogenesis of such neurological autoimmune diseases as multiple sclerosis and experimental allergic encephalomyelitis. It has also been pointed out that this cytokine exerts damaging effects on oligodendrocytes, on myelin-producing cells of the central nervous system, and on myelin itself (38,39), and that it plays a key role in augmenting inflammatory demyelination. A combination of TNFα and TGFβ has been reported to cause significant SC detachment and lysis in rat SCs (40) as well as in a human Schwannoma cell line (ST88-14) (Oliveira et al., in preparation for publication). Recent data have also shown the human SC cell line to express the TNF receptor and the TLR, the latter one in primary human SCs as well (Oliveira et al., submitted). In addition, apoptosis of these cells was induced by way of TLR2 stimulation via the lipoprotein 19kDa of M. leprae.

It is our understanding that the presence of TNFα both inside the SC and close by in the tissue milieu could lead to SC damage. To search for increased expression of TNFα in the tissue (dermis and epidermis), therefore, sounds reasonable in spite of its source. The potentially more logical contribution of macrophages, keratinocytes, endothelial cells, SC cells, neutrophils, and even T cells, in the local production and/or amplification of the TNFα response must be further pursued. Understanding the mechanisms involved in the elevated TNFα production in the surrounding tissue, and inside the nerves themselves, in conjunction with the ability of these cells to experience the effects of TNFα (cell stimulation, cytotoxicity, cell death) will signify a major breakthrough in solving the problem of the development of disabilities in leprosy.

In addition to the proposed direct effect of TNFα in neural injury across the entire spectrum of leprosy, re-emergence of antigen-specific CD4+ T cells has been considered to have a role both in protection as well as in immunopathology. CD4+ T cells are more abundant in the skin lesions of patients in reaction. In this regard, the anatomical location of CD4+ T cells containing cytotoxic granules has been described. Murine SCs have already been shown to function as APCs for CD8+ cytotoxic T cells in a major histocompatibility complex (MHC) class I-restricted, mycobacterial antigen-dependent man-

ner (41). The expression of HLA class II on human SCs has likewise been demonstrated along with the observation that IFNγ and TNFα action as well as *M. leprae* invasion resulted in the up-regulation of MHC class II by rodent SCs (42,43). Thus, SCs may be actively involved in the immunopathology of leprosy neuritis by presenting *M. leprae* antigens to cytotoxic T cells (44). As an alternative mechanism, expression of the neural cell adhesion molecule (N-CAM), also called CD56, on both target and effector cells has been hypothesized to be of importance. Besides NK cells, N-CAM expression has also been observed on some CD4+ T cells in relation to multiple sclerosis (45). These cells have been found to be able to kill N-CAM positive oligodendrocytes in an antigen independent manner (46). It has also been revealed that co-adhesion via other molecules (CD54 and CD11a) was essential in establishing target lysis. Such a mechanism may also be involved in leprosy neuritis since cells derived from inflamed neural tissue show increased N-CAM expression when compared to peripheral T cells.

How does *M. leprae* infection contribute to nerve injury?

It is clear that a crucial initial event in the neurological manifestations of leprosy is infection of SCs by the bacteria. Substantial progress has been achieved over the last five years regarding the mechanisms involved in the *M. leprae* attachment to SCs. It has been demonstrated that the bacteria bind specifically to the globular domain of the α2 chain of laminin-2, the most abundant isoform of laminin present in the basal lamina that covers the SC, and that this binding is necessary and sufficient for adherence to the cells (47). Moreover, the laminin receptor α-dystroglycan was shown to serve as the SC receptor for *M. leprae* (48). Subsequent studies have identified the cationic, histone-like protein (Hlp), also known as laminin-binding protein (LBP), and the phenolic glycolipid I (PGL-I), as potential bacterial adhesins involved in SC interaction (49-51). More recently, it has been observed that *M. leprae* rLBP also has the capability to bind several collagen isotypes, which indicates an expansion of this protein’s potential role in mycobacterial pathogenesis by way of its ability to mediate bacterial interaction with other cell types, such as epithelial and endothelial cells, as well as different base membranes (Lima et al., 16th International Leprosy Congress). The recent identification and characterization of *M. leprae* molecules capable of interacting with extracellular matrix (ECM) components raises interesting speculation as to the existence of alternative mechanisms by which these molecules, in addition to their roles as adhesins, could be contributing to the pathogenicity of nerve damage in leprosy. One
potential mechanism could be the capacity of these molecules to disrupt, via binding competition, the dynamic cross talk between axon-SC units and the surrounding extracellular matrix. In fact, a reasonable explanation for the recently reported demyelination effect of PGL-I (52) might be its capacity to disrupt the extracellular signals from the laminin-2 to the axon-SC unit. It is well known that the ECM present in the peripheral nerve system acts as an organizer of peripheral nerve tissue and strongly influences SC adhesion, growth, and differentiation as well as regulating axonal growth during development and regeneration (53). Moreover, the basement membrane plays a vital role in promoting the development of the myelinating SC and in stabilizing SC-axon interaction (54). Among its components, laminin-2 in particular seems to be involved in these processes by way of its signalling activity via the dystroglycan complexes present on the SC membrane (55-57). Demyelination defects are observed in laminin-2 deficiencies, such as congenital muscular dystrophy and the dy/dy mouse (58,59), as well as in mutations that affect the structure and/or signalling properties of the receptor complexes of basement membrane proteins (60). Likewise, it has recently been reported that a histone-like protein (Hlp) found in a number of pathogenic Streptococcus species contains stereospecific properties in the binding of basal membrane components of cardiac fibres and the kidneys. This same binding capability may also be related to the pathogenesis of bacterial-induced tissue inflammation (61). In this context, it seems reasonable to speculate that during the course of M. leprae nerve colonization, bacterial laminin-binding molecules, such as Hlp and PGL-I, accumulate within the nerve basal membrane. These molecules could possibly be released into the tissue during infection as a result of bacterial lysing (during chemotherapy and/or host immune response), and would, therefore, be responsible for the persistent inflammation and delayed sequelae observed as a result of M. leprae endoneural infection. It would appear worthwhile to investigate the validity of this hypothesis since it could represent a significant achievement in the understanding of progressive nerve damage in leprosy. In addition, intracellular events consequent to M. leprae interaction with the nerve have recently been investigated. Intracellular transduction signalling triggered during association of the bacteria suggests that tyrosine kinases and phosphatidylinositide-3 kinase signals are activated (Alves et al., 16th International Leprosy Congress). These signals are also triggered by the bacteria in human monocytes, and probably represent universal signals generated early on during phagocytosis (62). Additional results suggest that the intracellular niche of M. leprae has an aperinuclear localization in nonacidified vesicles (Lima et al., 16th International Leprosy Congress). The events occurring in SC/nerve co-culture systems are being addressed by two independent groups. The results of Hagge et al. (16th International Leprosy Congress) suggest, on the contrary to Rambukkana’s data (52), that M. leprae exercises no detrimental effects on SC function, reinforcing the idea that most of the neuropathy observed in leprosy is likely due to the aggressive immune response to the nerve infection. Moreover, preliminary data from our own group indicate that the effect of M. leprae on global gene expression in the SC line is implicated in very mild alterations as evaluated through the use of differential display polymerase chain reaction (PCR) and DNA microarrays (Tempone et al., 16th International Leprosy Congress).

It is clear that the study of lepromatous vs. tuberculoid forms is a common generalization of the infection and disease progression since clinical manifestations are the outcome of a very slow and complicated interaction between the bacterium and the host. In fact, leprosy is not a stable disease in any of its clinical forms. It is then possible that the outcome of M. leprae infection would in any case lead to the resolution of infection following the induction of IL-12, IL-18, TNFα and IFNγ cytokines. However, for some individuals, their genetic background would provide the scenario for a lower initial response (innate and/or specific immunity), allowing for subsequent bacterial multiplication and the establishment of different levels of non responsiveness that are present across the disease spectrum. Thus, leprosy exhibits a unique immunological pattern which is based on complex interactions between cells and secreted factors that may well represent a dynamic behaviour. From the point of view of clearing the bacteria, upgrading responses (reactions) might be considered to be beneficial. However, the inflammation in the tissue and the nerve may turn into permanent damage and disability, if not treated adequately. Therefore, it is of the upmost importance to pursue new and effective therapeutic interventions capable of promoting axonal regeneration in these patients.

**Major points for further investigation**

- **Mechanism(s) of Schwann cell injury in leprosy:** TNFα, cytotoxic T cell, apoptosis, N-CAM-N-CAM interaction.
- **The definition of tissue markers as indicative of nerve damage:** myelin components, ninjurin, adhesins, ECM components.
- **Schwann cell-M. leprae interaction:** demyelination, intracellular metabolism, interaction with ECM.
- **Establishment of therapeutic interventions for nerve regeneration:** MMP inhibitors, inhibitors of apoptosis, TNFα inhibitors, methylocobalmine, adhesin inhibitors.
References


51. Ng V et al. Role of the cell wall phenolic glycolipid-1 in the peripheral nerve predilection of *Mycobacterium leprae*. *Cell*, 2000, 103:511-524.


