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Leprosy Review

A journal contributing to the better understanding of leprosy and its control

British Leprosy Relief Association
LEPRA

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Leprosy Review is published by the British Leprosy Relief Association (LEPRA) with the main objective of contributing towards the better understanding of leprosy and its control. Original papers on all aspects of leprosy, including research, are welcomed. In addition, *Leprosy Review* seeks to publish information of educational value which is of direct benefit to the control of leprosy under field conditions, and hence to the individual patient.

From time to time the Editorial Board invites special articles or editorials from experts in various parts of the world, and gives consideration to the production of a supplement or special number devoted to a particular subject or theme of major importance.

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SYMPOSIUM ON MULTIDRUG THERAPY IN LEPROSY

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INAUGURATION

Opening addresses

I am very pleased to see that so many delegates from all over the world have come to Würzburg to take part in our Symposium on leprosy treatment and I would like to warmly welcome you all to our city.

To my understanding this Symposium will be an opportunity to present and to discuss recent results of chemotherapy research and related fields in an unconstrained but nevertheless very concentrated atmosphere.

I am very gratified to see that scientists who are supported by the German Leprosy Relief Association will have the chance to show that the German Leprosy Relief Association has been interested in finding effective medicines for a long time. It was above all Professor Joachim Seydel, Head of the Department of Chemotherapy Research and Head of the so-called 'Leprosy Research Crew' at the Institute of Experimental Biology and Medicine at Borstel, who by his worldwide connections with many of our lecturers decisively contributed to the shaping of our programme.

I very much hope that besides making a detailed inventory for which Session I 'Clinical Aspects' shall be especially useful, we will also be able to present and discuss the latest techniques and results from renowned scientific institutes.

Emphasis will be laid on the fact that completely different ways may lead to the same goal, that is effective control of chemotherapy; Session II therefore has the heading 'Test Models for the Effective Control of Chemotherapy'. Imagination will take the floor in Session III when 'Developments and Future Aspects' will be discussed.

Enough time will be left over between the Sessions to have personal talks and to become acquainted with each other, if this should still be necessary in this circle. It will no doubt be necessary to establish contacts between, well I may say so, laboratory scientists and field scientists who should discuss their individual needs and requirements.

We also wish, however to get to know scientists from the University of Würzburg who direct or work in institutes which have or could have connections with leprosy work. We hope to be able to make the first step here towards closer co-operation between the German Leprosy Relief Association and the University of Würzburg. For that reason I am very pleased that one of the members of the University of Würzburg, who is also one of our Medical Advisors, namely Dr Klaus Fleischer, will give the keynote address today.

We are above all very thankful, Mr Vice President, to the University for giving us the chance to have our Symposium here in these rooms.

Apart from encouraging you to use the following three days for the benefit of scientific matters of leprosy therapy and of the patients suffering from this disease, I would also urge you not to forget to enjoy the beauties of our city, the Lord Mayor of which I cordially welcome here. The GLRA members of staff will be pleased to do their very best to make you feel comfortable and please do not hesitate to contact Frau Rößler, Frau Hajek or me, if there is any problem.

Finally I wish all of us three successful and pleasant days.

Thank you so much!

DR HORST FRANAK

Despite the considerable advances and successes achieved by medicine in our age, leprosy, which has been known to us from biblical times, still remains one of the scourges of mankind. Even today, more than ten million people in the world are afflicted with the disease, mainly, of course, in countries in the Third World. Leprosy does not only inflict physical decay and torment on the sufferer, but, as the German term for the disease, *Aussatz*, indicates, it also means exclusion from society. It is therefore all the more significant that, for the first time in the history of the fight against leprosy, the rigorous implementation of a treatment programme has succeeded in eradicating the disease from a whole area, namely Malta, formerly the last stronghold of leprosy in Europe. To mark this occasion, this symposium is being held here in Würzburg, the seat of the German Leprosy Relief Association, with the aim of providing an opportunity for the exchange of experiences concerning the use of multidrug therapy, which was so successfully employed in Malta. I am very pleased that Würzburg has been chosen as the venue for this symposium, not only because the German Leprosy Relief Association has its seat here, but also because the Würzburg Faculty of Medicine can look back on a far from undistinguished past. Names like Siebold, Döllinger, von Koelliker, Rinecker, Rindfleisch and Virchow testify to the high standard of the work carried out by the Würzburg Medical Faculty. At the same time such prominent figures in the field of medicine also serve as examples for all those who have committed themselves to serving man with all his ailments, illnesses and diseases.

In connection with the theme of this symposium, we would do well to remember that the foremost task of medicine is to alleviate human suffering. Today it is particularly important to bear this in mind in our affluent society with its growing tendency to turn medicine into soulless technology and inhuman machinery. The struggle against leprosy may serve here as an example of how the doctor can act as a human being rather than functioning like a technician, even though, of course, he cannot perform his tasks adequately without having recourse to the results of technological and pharmacological research. Successes in the fight against leprosy do not necessarily hit the headlines in the media, but

they are, nevertheless, at least just as important as a heart transplantation. Such success as achieved by multidrug therapy demonstrates to the world how highly beneficial the results of medical research can be for humanity.

I am extremely impressed by the fact that medical research scientists and doctors from so many countries in the world have joined together to achieve this common success in the fight against leprosy and I am proud that so many have found it possible to attend this conference here in Würzburg.

I could think of no better wish for myself or one of my successors that to be able to announce here in Würzburg in the foreseeable future that another country has finally been freed of the scourge of leprosy.

I should like to extend a warm welcome to all those participating in this symposium and to wish the conference every success.

PROF. DR. DR. HANS-ACHIM MÜLLER

A cordial welcome in Würzburg to all participants in the 'Symposium on Multidrug Therapy in Leprosy'. For the first time a new drug regimen puts mankind in the position to not only stop leprosy, but to heal it finally. This is a milestone in the medical development of an efficient therapy against leprosy.

I wish to congratulate all scientists who contributed to the development of this therapy to their outstanding achievement and I thank above all the German Leprosy Relief Association that, by using this new treatment, will continue to perform its beneficial work which, originating from Würzburg, brings help to the poorest of the poor all over the world.

Big talks and nice words do not change anything. But there are the scientists who fight this cruel scourge of mankind by making new discoveries. And there are also the many individuals who by giving donations and assistance, virtually show solidarity with our leprosy-stricken fellow brothers who often live in unbelievable misery.

DR KLAUS ZEITLER
(Translated by Karin Rößler)

Tribute to Professor Dr Enno Freerksen and Dr Harold Wheate

One of the reasons of this Symposium is that the German Leprosy Relief Association wants to pay tribute to Professor Dr Enno Freerksen who may be

considered as a real pioneer of the development of new and effective drug regimens against leprosy. He paved the way for new approaches and developments without which this Symposium would not take place.

On the occasion of its General Assembly in November 1985 the German Leprosy Relief Association already honoured Professor Freerksen in an official way. I should like to seize the opportunity now to express to him once more the heartfelt thanks and deep appreciation of our association and of leprosy patients all over the world.

We also wish to pay tribute to a good and old friend of our association. In consideration of his long and outstanding engagement in leprosy work and his fruitful work as the Secretary to the Medical Commission of ILEP, the German Leprosy Relief Association thankfully bestows this document to Dr Harold Wheate.

HUBERT COUNT BALLESTREM

Reply from Professor Dr Enno Freerksen

Let me thank you for the honouring words which I accept for my co-workers at the same time. First of all, I pass thanks to Mrs Rosenfeld with whom I have been studying these problems for decades, and furthermore to the great number of co-workers abroad, especially to those who are directly engaged in today's colloquy: Dr Alvarenga from Asunción and Dr Depasquale from Malta. Both will report today on their results.

There is no other way than giving honours always to persons. But it has to be asked what advantage patients have from our work, and where the imperfections are which directly concern practice but only can be removed by scientific work. Please, accept some short remarks in this connection.

In scientific studies on chemotherapy a lot of factors must be considered but effectivity, tolerability, practicability, and acceptance will always remain as the big four on which success depends for each therapy. If only one of these four is missing, there cannot be an effective treatment. Even high effectiveness does not help when there is no tolerance. The best therapy is also worthless when not being applied. And all is in vain when medication is refused by the patient—indifferent by which reasons.

Although the fixed combination Isoprodian–RMP includes four drugs, the daily dosage does not exceed 2·000 mg for adults. This is of great importance because of the compliance. The medication does not require a differentiation between multi- and pauci-bacillary cases with the consequence of saving time and money and avoiding faults. By including RMP into the fix combination, such a

high level of safety could be reached that no more medical supervision is necessary than for any other outpatient treatment.

The application is really simple: 2 tablets in the morning and 2 in the evening—nothing could be more simple. So, we have at hand a highly effective, easily applicable and well tolerated therapy which can be used everywhere for out-patient as well as for clinical treatment.

In spite of this favourable situation, we still need new and more therapy regimens. There cannot be a uniform medication for the whole world. Any progress in science would be cancelled by this. But even independent to this point—a physician needs alternatives in order to adapt treatment to individual and regional requirements. In addition, we should have courage to expand our aims. While an effective treatment is given to as many patients as possible—with flexibility and alternatives—we must try to reach in days or weeks what nowadays takes months or even years. To search for new substances therefore will still be one of our most important tasks for the future. My personal opinion is that due to practicability, acceptability and safety fix combinations will be a great help.

But not only are more drugs needed. We also need more clearness in some basic questions regarding leprosy (i.e. methods for differentiation of viable and nonviable bacteria, significance of various types of bacteria, BI and termination of treatment) and so on.

Urgently required are clear definitions for some terms which obviously are used with quite different meanings: what means 'eradication', what is a 'relapse'? Are there any relations between leprosy reactions and chemotherapy (and if so, which)? What is the sense of 'generation time' considering the specific mode of multiplication of mycobacteria? Of course, this catalogue of questions could be completed. It seems that some discussions up to now remain unproductive because of their indistinctness of definition.

Leprosy therapy in principle was pushed forward by means of the introduction of combined therapy (MDT) and by carrying out the Malta eradication programme. Leprosy could be taken out of its isolation by transforming treatment of leprosy and tuberculosis to a 'chemotherapy of mycobacterioses'. This will be demonstrated with the great number of cases of the Paraguay project.

Today's colloquy will be an important contribution in this direction. I am really glad that it is held in Würzburg as I dare say that there is no other organization to which we owe so many thanks than to the German Leprosy Relief Association.

Keynote address

Not as a scientific leprologist but as a medical practitioner concerned with the health of people in countries where leprosy is but one health problem among

many, I want to make a few comments on multidrug therapy in leprosy as compared with the hitherto commonly practised monotherapy. I then reflect on the relationship between research fellows and all those in the field who have to apply the research findings. Finally, I should like to remark briefly on the integration of MDT into the general health services.

We all are eagerly looking forward to the reports of this symposium which will present the advantages of MDT compared to monotherapy. These advantages are so evident and probably so convincing for all of us that there will in principle be little doubt on the superiority of MDT and its success.

We shall, however, notice in the forthcoming discussions that MDT is not just a matter of combinations and doses, but that MDT alters the relationship between the healer and the sick substantially. MDT in its relative complexity considerably raises the level of preconditions required to implement it. To apply MDT successfully an advanced standard of training is wanted to keep pace with the new developments, e.g. those reported here, a standard of organization which only a percentage of centres have presently at their disposal, and certainly much more money would be needed—but we don't want to talk about money as guests of GLRA.

Leprosy therapy by MDT happens at present mostly on the level of the individual doctor/patient relationship—a level which is well above the paramedical worker-level which has been established so successfully with monotherapy. When we exclude for the moment a series of excellent pilot projects in Africa, Asia, and the Americas—many of whose masters are among us and to whom I pay respectful tribute—MDT is still reserved for the wise doctor who knows what is good for his patient, who has learned for many years to study, to compare and to question. The tricky problem when to end MDT is still left entirely to the doctor, as he alone can bear the responsibility on the basis of his experience and laboratory criteria, not definitely defined so far.

Due to the very special and detailed knowledge which is needed to apply MDT, there is the real danger that the gap may widen between the fully trained doctor and the paramedical staff—a gap that was reasonably bridged at the time of monotherapy. If the distance becomes too great this may result in a reduced service rendered to the patients, the majority of whom are, and will be, served by field staff.

The relationship between the theorist and the practitioner, as well as the relationship between those who favour monotherapy and those using MDT already deserves—I believe—our particular attention. We have got to give the same intensive priority and passion to the training of all who apply MDT as we give to its research.

We don't want to wait for a new generation of fieldstaff, do we? We need to rely on the old and experienced staff, who will, however, require further extensive training.

Training again which ought to bridge the gap between the doctor and the

auxilliary staff, training which can really be put into practice by the trainees, such a training requires appropriate language. We academics have to make efforts to translate the aim and methods of MDT into a language which makes these clear and at the same time stimulates enthusiasm and co-operation among leprosy workers. The technique of translation of scientific results into practical instructions needs to spread from training centres like ALERT to the peripheral centres and it needs to be adapted to local contexts. Paramedical workers know how their patients react to monotherapy and by experience they are suited best to overcome weariness and defaulting. Qualities which are also needed badly for the implementation of MDT.

The field staff doctors and paramedics alike depend on an MDT which is: relatively simple to apply; somehow standardized; as safe and reliable in the hands of leprosy workers as possible; convincing in its effects both for the workers and patients; and suitable for supervision and evaluation.

The claim for simplicity is paramount—we cannot do without it under the difficult conditions of urban as well as rural leprosy work. Not only the MDT-regime, but the entire operational set-up has got to be so simple that it will work under adverse conditions in the hands of paramedical workers. I just want to mention some of the possible stumbling blocks for them:

Are they certain about the criteria to define whether the disease is active or inactive?
Do they recognize reactions and drug induced side-effects?
Is it necessary to stick rigidly to fixed dates of tablet administration without allowing for circumstantial variations?
How many control smears are adequate in a certain regime?
Does the feedback in the so called 'smear chain' from the laboratory back to the fieldworker happen in a reasonable time?
What about the higher demands made on the recording system?
And last but not least, are doctors able to provide the intensified medical supervision that is needed?

In the laboratories and units where you study the effects and interactions of old and new compounds and the clinical aspects of MDT with refined techniques and statistical methods, you use 'high tech'. That means much more than photometers, HPLC and data-processing; it comprises the entire highly specialized ways and aims of leprosy research without the findings of which MDT would just not be. Out there in the field not only electrical power, gear (equipment) and know how are missing, the entire setting asks for an approach which I want to call with respect 'low tech'. Now 'low tech' is not a 'by chance' second-class method', primitive but cheap—it is rather an intelligent development of existing technically simple methods. Such a solution of problems adapted to the situation, needs an input of purposeful health education—and an analysis of the economic proportions (terms of references), e.g. a detailed knowledge of

how workers and populations accept the new therapy offered. Adapted appliances like solar or battery operated microscopes and standardized smear-reading fall in with it. Thus the success of MDT cannot be reduced to the simple question 'What does a drug-regime effect?' It is dependent on the answers to questions like:

'Does it work under given circumstances?'
'How good is the in-service training?'
'How good are the controlling eyes?'
'How can results be evaluated?'

The considerations of the user of 'low tech' are just as important as the aims of the researcher for 'high tech'. The cause of the fieldworker asks not only for our tolerance but our full intellectual and emotional support as their lobby is weak compared to the one of the researchers. All of us here, biologists and doctors, are the servants of the practitioners who have got to do the job on the spot.

The fieldworkers are faced with a situation which makes it more and more difficult to keep up a vertical leprosy service. It meets not only the philosophy of primary health care but our own conviction of a modern leprosy service too, that it should become integrated into the general health services. MDT demands, on the other hand, detailed medical knowledge more than ever before from all workers so that the leprosy units might be reluctant to integrate MDT in order not to water down their special services. Leprosy patients, however, are part of their urban and rural communities and rightly claim their integration into the health and educational opportunities of their region.

We bio-medical researchers would, I think, be well advised to discuss and investigate the possibilities of applying our research results with epidemiologists and educationalists. This would probably lead us to extend our research interests to problems of basis health care, the application of leprosy treatment within these services and the organization of programmes and training of suitable personnel.

The aim is, colleagues—or is it not—to let the people suffering from leprosy all over the globe participate fully in the excellent progress that you have made in leprosy therapy.

Thank you for your patient attention.

DR KLAUS FLEISCHER

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Abstracts

SESSION I. CLINICAL ASPECTS. *CHAIRMAN:* D L LEIKER (THE NETHERLANDS)

1 The Malta experience—rifampicin and Isoprodian combination treatment for leprosy. G Depasquale

Since 1972, a total of 247 patients have been treated with a rifampicin–Isoprodian combination for a specific period of time as determined by individual clinical and bacteriological progress, and were subsequently controlled periodically for the possibility of relapse. The age, sex, and disease type distribution is demonstrated and the tolerability to treatment and results are discussed. Practically no patients have relapsed, and to date, 145 patients have been under regular post-treatment control for over 10 years, while a further 57 patients have been controlled for over 5 years.

2 Epidemiology of leprosy in Malta. D L Leiker

A reconstruction of the distribution and the trend of leprosy in Malta since 1900, based on notifications to the Ministry of Health, records of leprosy hospitals and patient records. A decrease in the incidence since 1900 was found. The most rapid decrease occurred in suburban areas, followed by rural areas. In the last decades most of the new cases occurred in peripheral rural villages, 'at the end of the road'.

Preliminary results of treatment of leprosy in the Netherlands are available with daily rifampicin, and dapsone, and clofazimine on alternating days. About 400 patients, one third lepromatous and borderline-lepromatous, mostly pretreated with a single drug, were treated for 1 year with the drug-combination and thereafter released from treatment and kept on observation.

So far, 3–5 years after release from treatment, no relapses were found.

3 First evaluation of the Malta leprosy eradication project. D L Leiker

Independent assessment of classification, treatment history and the bacteriological and histopathological status of leprosy patients in Malta 4 years after release from treatment with rifampicin and Isoprodian.

Assessment of side-effects of the drug-combination and causes of death.

4 A follow-up investigation of the Malta-project, 1983 and 1986. W H Jopling

The first follow-up examination, carried out in April 1983, included 116 multibacillary leprosy patients who had completed multidrug therapy (MDT), the majority having commenced MDT in 1972, and the minority subsequently. Length of treatment varied between 5 and 89 months, and side effects were mostly mild. No signs of clinical relapse were found, but 36 patients had positive skin smears; 26 had granular bacilli alone, and 10 had scanty 'solids'. Details of these findings constitute the first part of this report.

The second follow-up examination will take place in the early part of April 1986 in order to discover if any of these 10 patients show clinical or bacteriological evidence of relapse, and these findings constitute the final part of this report.

5 Report of the joint leprosy/tuberculosis project in Paraguay. A E Alvarenga

On the basis of the agreement between the government of Paraguay and the German Leprosy Relief Association (DAHW), a programme is being developed in this country for the eradication of leprosy and tuberculosis. This joint programme started in 1979.

For programme execution a joint leprosy/tuberculosis organization was created under the centralized management of the directorate of the government leprosy service.

Programme implementation is being effected through the existing health infrastructure. This implies that the programme directorate has to implement the programme through two administratively and organizationally different systems. The leprosy programme has a vertical management whilst the tuberculosis service is of a horizontal nature with several administrative levels responsible for the decision making process. This situation requires a rather elaborate type of coordination in order to achieve a uniform programme implementation.

In both programmes the same combined chemotherapy is being used, namely Isoprodian-RMP. The introduction of this regimen, because of its rapid therapeutic action, has made a significant impact in the fight against both of these endemics.

The anti-leprosy programme was developed in two stages:

1. The start of the project in Asunción and its surrounding smaller towns. About one third of the national population is concentrated in this area.
2. During the second stage, which is now in progress, the programme is being gradually extended to the population of the interior. To this end, the available infrastructure as well as the leprosy prevalence are being taken into account.

Until 31 December 1985 some 1623 cases of leprosy were admitted by the programme. This represents 32% of the total registered cases in the country. Of these patients, 797 (49%) terminated treatment whilst 685 cases (42%) are actually passing through the different phases of the therapy.

In 2 patients only, following 2 years of post-treatment observation, therapy had to be reinstated because of clinical reactivation.

The execution of the tuberculosis programme also underwent a gradual development. The start was in Asunción and its neighbouring populations as well as in settled groups of indigenous populations living in the Paraguayan chaco.

In 1980 the TB-programme had a population coverage of approximately 1·6 million. The principal objective of the programme is to reduce as rapidly as possible the TB morbidity and mortality rates. For this purpose a rapid extension of the programme to obtain the widest possible population coverage is necessary—subject to the availability of resources.

Medical attention to TB-patients is being provided by the government health centres. This type of attention is integrated into the general health service.

For programme implementation the government TB service also can count on the collaboration of several voluntary organizations providing assistance to the indigenous populations. Frequently these organizations employ trained lay workers for the task.

The short-term therapy with Isoprodian-RMP is showing very good results indeed, so much so that in some indigenous populations tuberculosis has been practically eliminated.

To the vast majority of patients anti-TB drugs are issued ambulatory. A few serious cases only are hospitalized in special institutions.

During 6 years of programme activity, within the corresponding areas, 5853 tuberculosis patients were detected, or in other words, some 25% of the total estimated number (22,812) of cases in the country. In detail:

TB cases detected within 6 years	5853
No. cured	2835
Abandoned treatment	1261
Left the area	429
Died	385
Actually on treatment	943

The high number of abandoned cases results from the fact that we have to adapt the old organization to the new requirements.

The introduction of the combination Isoprodian-RMP constitutes a most important advance in the fight against leprosy and tuberculosis, principally this is because of its rapid action and outstanding efficiency in the treatment of these two diseases. The period of treatment is vastly shortened with all its positive consequences derived from this progress.

In the light of these promising results, the ambitious goal of planned disease eradication which has no parallels in other programmes, imposes the need for continuing the ongoing programme until its final realization. To fulfil this attainment it is imperative that the joint TB/leprosy programme can count on the continuation of the invaluable support of the German Leprosy Relief Association (DAHW). This great challenge has found the backing of the Parguayan Government and its people almost from the onset of the programme.

6 Combined treatment schedules for leprosy. A prospective randomized multicentre study. Clinical results. M Dietrich, Clinical Study Group*

Confirmed cases of lepromatous or borderline lepromatous patients were randomized to receive one of the three following drug regimens: (a) DDS 100 mg/day, (b) DDS 100 mg/day + Rifa 600 mg/day, (c) Rifa 600 mg/day + Isoprodian, 2 tablets/day. A complete physical check-up, basic laboratory test, skin smears and histology were done before treatment and at regular intervals during the 3 years of treatment. Prior to chemotherapy, a DDS resistance test was performed and in case of DDS resistance the patient was put into group D which is equivalent to group C. 302 patients were randomized in five different centres: Freetown (Sierra Leone), Karachi (Pakistan), Bombay, Madras and Chetput (India). The study design was to treat patients for 3 years and to have a follow-up period of 5 years. Presently there are still 245 patients in the study, 69 in Group A, 90 in Group B, 86 in Groups C and D. There is no statistical difference concerning the variables sex, age or disease classification (BB or LL) in the three groups. We report the results after 3 years of treatment. Of the 102 cases we evaluated, 87 show a regression while 14 patients were clinically classified as stable leprosy. The bacteriological index as well as the acid-fast bacilli in the skin biopsy decreases by

* Clinical study group: M Dietrich, M Aschhoff, G D Burchard, T Chiang, V Devanbu, S Engelhorn, H Feldmeier, R Ganapati, W Gaus, P P Irudayaraj, J Jayakumar, P Kern, U Laukamm-Josten, M Peters, R Pfau, J Rangaraj, M Rangaraj, C R Revankur & R Wabitsch.

about the same amount in all treatment groups per year. This preliminary evaluation shows no difference in therapeutic response in combined as well as single-drug therapy. The clinical, bacteriological and histological parameters have clearly improved. The three drug regimens were tolerated well, and there was no difference in side effects as judged by GOT, GPT, B.U.N., and haematology serial examinations. As none of the three drug regimens seemed to be superior, the evidence of relapse and/or the development of DDS resistance in the follow-up period may prove to be the crucial criterium for final judgement.

7 The impact of multidrug therapy drug implementation in the Tanzania National TB–Leprosy Programme. H J Chum

8 Effect of clofazimine and dapsone on rifampicin (Lositril) pharmacokinetics in multibacillary and paucibacillary leprosy cases. J M Mehta, I S Gandhi, S B Sane, M N Wamburkar

A comparative pharmacokinetic study of Lositril (rifampicin) was carried out in 6 multibacillary and 12 paucibacillary leprosy cases. The type of leprosy had no significant effect on rifampicin pharmacokinetics.

The effect of dapsone and clofazimine when given separately and in combination was studied on rifampicin pharmacokinetics in each group of 6 patients. Within group comparison revealed that clofazimine reduced rifampicin absorption significantly ($P < 0{\cdot}01$) and prolonged the time to reach the peak serum concentration ($P < 0{\cdot}01$). Since MCR and Ke were also reduced significantly in RC group, as compared with RDC group ($P < 0{\cdot}02$ and $P < 0{\cdot}05$ respectively), no significant alteration was seen in overall Auc and Cmax, although $t0{\cdot}05$ was increased significantly ($P < 0{\cdot}02$) in RC group.

Dapsone alone did not produce any significant alteration in rifampicin pharmacokinetics parameters, while dapsone with clofazimine reduced rifampicin 1 h serum levels ($P < 0{\cdot}05$) and Auc ($P < 0{\cdot}05$) significantly.

Of the three groups, except RC group, both RDC and RD groups were homogenous Ka, avd, Cmax and Auc/$t0{\cdot}5$ ratio of RC group were significantly different from those in RD group. While Ka and avd were significantly less ($P < 0{\cdot}05$ and $< 0{\cdot}001$ respectively) and Cmax and Auc/$t0{\cdot}5$ ratio were significantly more ($P < 0{\cdot}01$) in RC group. Since clofazimine reduced rifampicin absorption, the difference in Ka and tp became more significant in the post-regimen phase ($P < 0{\cdot}01$).

9 Some clinical impressions of multidrug therapy in leprosy. J M Mehta

The Poona District Leprosy Committee is running two leprosy control projects, one in the urban area of Poona and the other at Solapur. In these projects, by the end of December 1985, 4532 leprosy patients were given multidrug therapy. Of these, 1828 patients are multibacillary while 2704 are paucibacillary.

The standard regimen recommended by the WHO Technical Report Series 675 is being followed for multi- as well as paucibacillary cases. The patients who have maintained regularity in treatment have shown clinical as well as bacteriological improvement. They did not show major side-effects/toxicity. A detailed analysis of the data is being undertaken. However, a few cases in whom interesting clinical findings were observed are reported here.

In 21 multibacillary cases, the bacteriological index started falling initially and after it dropped to 1·0/1·5, it remained static, even after continuing MDT though they showed definite clinical improvement. The pigmentary changes and the ichthyotic changes seen during clofazimine therapy persist for more than 2 years after stopping clofazimine. In a few patients, pigmentation due to clofazimine therapy is not pronounced, though supervised clofazimine therapy is confirmed. The reason for this is not known.

In 20 paucibacillary patients, those previously treated with dapsone monotherapy and later put on MDT did not show much clinical improvement in respect of the patches, while clinical response in new untreated paucibacillary patients treated with multidrug therapy is excellent, as observed in the disappearance of the patches. It is the observation of some of our colleagues that if MDT is discontinued after 6 months, patients report back within 1 year with relapse in the form of new patches.

10 Experience with MDT—clinical, operational and managerial implications. M Rangaraj

11 Clinical problems in the initiation and assessment of multidrug therapy. M F R Waters, D S Ridley & Marian J Ridley

The introduction of multidrug therapy is essential to overcome major problems of dapsone resistance, both primary and secondary, and hopefully also of compliance and of microbial persistence. The division into multibacillary and paucibacillary leprosy, as postulated by WHO, depends on both accurate clinical classification and smear taking and reading of a high standard. In this paper, we shall discuss some of the classification problems which we have experienced, as exemplified by smear-negative, neural BB and BL leprosy.

We shall also discuss the assessment of the results of treatment of paucibacillary leprosy, as great difficulty is often experienced in distinguishing between bacterial relapse of treatment from late reversal (upgrading or type 1) reactions. We have observed the latter to occur as late as 3 years after commencing (and continuing on multidrug) therapy in BT leprosy.

12 Preliminary evaluation of the effect of WHO/MDT on disabilities in leprosy patients in Malaŵi (Central Africa). J Pönnighaus & G Boerrigter

In a preliminary analysis of disability rates at registration, at completion of treatment and at 1 year after completion of WHO/MDT, we have shown that the percentage of patients treated with WHO/MDT who developed new or worse disabilities (5·7%) was similar to the percentage of patients treated with dapsone monotherapy who developed new or worse disabilities (2·7–6·1%).

On the other hand, review notes of the field staff appear to indicate that a higher percentage of patients (52%) recovered lost functions during and after WHO/MDT than during dapsone monotherapy (19–28%).

13 The use of MDT in three western regions of Nepal. P G Kalthoff

It has been proved that, even under very difficult field conditions like in Nepal, MDT can be introduced in the field if there are detailed instructions available for the PMW, together with

sufficient training provided. In addition, supervision needs to be done, particularly at the beginning to introduce the new habits properly, as well as periodically afterwards to make sure the standard is kept. We are hoping that the new patient recording system will help to improve the standard further, and particularly to allow us precise evaluation of the programme in the future.

14 Operational aspects of the implementation of multidrug therapy at ALERT, Ethiopia. M Becx-Bleumink

The ALERT Leprosy Control Department is responsible for leprosy control in Shoa Administrative Region. This region is centrally located in Ethiopia; it covers an area of about 85,000 sq. km, with a population of 8·75 million. The region is divided into one urban and eleven rural districts.

Leprosy diagnostic and treatment services are given in 292 centres; 60% of these are attached to general medical services and 40% are leprosy clinics which have been established in those areas where a general medical service does not exist yet. About 50% of the centres are accessible by car during the whole year. Multidrug therapy (MDT), according to the WHO recommendation of 1981, was introduced in January 1983. Paucibacillary patients are treated for a period of at least 2 years and until their skin smears have become negative.

In October 1983 a 'Manual for implementation of Multiple Drug Therapy in Ethiopia' was finalized; a second, revised, edition of this manual became available in February 1985.

During 1983 MDT was introduced in two rural districts (64 clinics): during 1984 in one urban and two rural districts (48 clinics) and in 1985 in two rural districts (61 clinics).

Prior to the introduction of MDT the leprosy control services were reorganized and intensified. This includes clinical and bacteriological examination of the patients under treatment, release from treatment of those patients who were considered of having received sufficient treatment with dapsone monotherapy, introduction of new recording and reporting systems, health education campaigns in the clinics and the communities, redefining of tasks and training of all cadres of staff involved.

During the period 1 January 1983 to 1 July 1985 3401 multibacillary patients and 2759 paucibacillary patients have been put under MDT. By the beginning of July 1985, 740 multibacillary patients and 2285 paucibacillary patients had completed their course of MDT. Until July 1985 one BT relapse has been diagnosed. Evaluation of the results of the treatment is done by way of cohort analysis: Of the 2543 paucibacillary patients who started MDT during the period 1 January 1983 to 31 December 1984, 2297 patients (90·3%) completed their course of MDT within a period of 9 months; 202 patients (7·9%) had their treatment discontinued because of irregularity of attendance; 22 patients (0·5%) had been transferred to a non-MDT area; 12 patients (0·5%) had died and 11 patients (0·4%) continued the treatment after 9 months.

During 1986 the MDT programme will be further expanded to two rural districts. We have planned that by 1990 the whole region will be covered with MDT.

During the period July 1982 to July 1985 the number of patients under chemotherapy in the region has decreased from 20,908 to 10,507. This decrease is mainly due to the release from treatment of over 5500 patients after dapsone monotherapy and the introduction of MDT.

Patients who have been released from treatment are instructed to attend regularly for follow-up examinations. So far 25–30% of the patients came for the appointed follow-up examinations.

About 3500 patients who have been released from chemotherapy since July 1983 continue to need care because of disabilities.

We have experienced that proper planning and organization of the MDT programme, including preparation of a detailed manual, are of extreme importance in order to guarantee proper implementation and evaluation of MDT. Workshops for the staff involved in MDT are conducted

at regular intervals. Priorities for future leprosy control in those areas where the number of patients under treatment has decreased to a large extent have been defined.

We are in the final process of making preparations for the field studies in one of the MDT areas; one study on the incidence of relapses, one on reactions during MDT and during the first year after release from MDT.

Problems we experience in the MDT programme will be discussed. These are especially:
Problems related to the severe drought and the acute shortage of public transport,
analysis 'by hand' of the many data which are essential for evaluation of MDT and the leprosy situation over the years.
The differentiation between relapse and reaction in paucibacillary patients. This is becoming an increasing problem.

15 Combined chemotherapy of multibacillary leprosy of 6 months duration. T Saylan, N Onsun & S R Pattyn

A treatment regimen of 6 months duration and composed of 2 weeks daily RMP (600 mg), PRO (500 mg) and DDS or CLO (100 mg) followed by 24 weeks RMP (600 mg) once weekly and daily PRO (500 mg) and DDS or CLO (100 mg), was administered to a group of 72 MB patients (45 new cases and 27 cases treated previously). Nineteen patients could be followed for 2 and 3 years after the end of therapy. No relapses were observed. The confidence limit of this result is 17·5.

16 The future of leprosy in the Dominican Republic and experiences made with MDT. D Martinez Cruz

SESSION II. TEST MODELS FOR THE EFFECTIVE CONTROL OF CHEMOTHERAPY FREE COMMUNICATION. *CHAIRMAN:* A M DHOPLE (USA)

17 The use of rodent models in assessing antimicrobial activity against *M. leprae*. R H Gelber

The ability of antimicrobial agents to prevent multiplication of *M. leprae* in the mouse foot pad remains the only generally acceptable means of assessing their potential for clinical application. The first screening technique to be utilized, the 'continuous method', employed uninterrupted treatment from the time of footpad infection, initially with the highest concentration of drug tolerated, orally if possible. Unfortunately, this technique did not distinguish between purely bacteriostatic agents and those with bactericidal effects. Thus a method termed the 'kinetic technique' was developed wherein drugs are administered from day 60 to 150 following foot pad infection. Agents that inhibit growth only during administration are considered bacteriostatic and those that appear to limit multiplication even after treatment has been discontinued are considered bactericidal. More recently the 'proportional bactericidal technique' for more direct assessment of *M. leprae* killing was developed. By this technique mouse foot pads are inoculated with 10, 100, 1000 and 10,000 *M.*

leprae, mice treated for the first 60 days, and foot pads harvested and *M. leprae* enumerated 1 year later, a time sufficient for any surviving *M. leprae* to multiply. This method allows for a quantitative assessment of bactericidal activity and comparison of the relative killing potential of various agents. The application of these methods will be reviewed and limitations of their utilization detailed.

Because there are no well established means of predicting which antimicrobials will be active against *M. leprae*, our strategy in selecting agents for testing primarily involves selecting drugs found useful against cultivable mycobacteria and those that act at loci thus far unexploited in the therapy of leprosy. Because only antimicrobial agents with some bactericidal potential merit further investigation, our initial screening efforts utilize the kinetic technique at maximally tolerated doses. We further study active agents by the proportional bactericide technique and, at times, at lower concentrations and frequencies of administration. In recent years we found a number of cephalosporins, cephamycins, doxycycline and erythromycin inactive. We have had variously promising findings with cycloserine, aminoglycosides, certain dihydrofolate reductase inhibitors, cephradine, amoxicillin/clavulanic acid, ciprofloxacin and especially minocycline. The results and the status of our ongoing studies will be detailed. In addition, the ability of various dietary concentrations of dapsone, rifampin, clofazimine and ethionamide to kill *M. leprae* as assessed by the proportional bactericide technique will be presented. The possible implications of those results to the therapy of leprosy in man will be discussed.

The neonatally thymectomized Lewis rat (NTLR) infected with *M. leprae* presents a situation analogous to human lepromatous leprosy, wherein viable persisting *M. leprae* remain despite therapy with all regimens of dapsone and rifampin previously utilized. We have been studying the effect of other combination chemotherapy regimens in NTLR to assess their relative efficacity to prevent persisters in established NTLR infections of 10^7 *M. leprae* or more. Regimens include: 1, larger doses of rifampicin alone and combined with dapsone; 2, clofazimine and rifampicin; 3, dapsone and ethionamide; and 4, rifampicin and ethionamide.

The results of these studies to date will be reviewed.

The standard means of laboratory monitoring of clinical trials in lepromatous leprosy have been the determination of the viability of 5×10^3–10^4 *M. leprae* obtained from biopsy material, usually skin, in the mouse foot pad. Attempts at monitoring trials utilizing thymectomized/irradiated mice, neonatally thymectomized Lewis rats (NTLR) and nude rats, as well as utilizing larger inocula in normal mice and some of these rodent systems are being employed in our laboratory and elsewhere. The current status of these studies will be reviewed.

18 Limited *in vitro* multiplication of *M. leprae*—application to screening potential anti-leprosy compounds. A M Dhople

Inability to cultivate *Mycobacterium leprae* (*M. leprae*) *in vitro* has been a major bottleneck in leprosy research. Today, the leprosy bacillus remains the only bacterium causing disease in man that has not been cultured *in vitro* and until this is achieved, all studies on leprosy will remain at a serious disadvantage compared with other human bacterial infections. We have initiated studies in this direction and the preliminary findings are presented at this symposium.

The studies done so far by other investigators, though unsuccessful, dealt mainly with microscopic and/or macroscopic growth of *M. leprae* in a given medium. But we have adopted three biochemical indicators to follow the fate of *M. leprae* incubated in a given medium. The first one is adenosine triphosphate (ATP) content of *M. leprae*; because of its ubiquitous distribution, the quantitative measurement of this compound is a promising method for detecting and quantitating microorganisms. The second one is deoxyribonucleic acid (DNA) content of *M. leprae* because of its role in cell replication. The last one is the uptake of (3H) thymidine by *M. leprae* because of its role in the synthesis of DNA and also because of the evidence available on its relationship to

viability of *M. leprae*. In our studies, we have demonstrated that 17% of the total (3H) thymidine uptake by *M. leprae* is due to its incorporation into *M. leprae* DNA. Furthermore, we have observed that *M. leprae* possesses thymidine kinase but not thymidine phosphorylase, suggesting that thymidine is converted to thymidine monophosphate and thus, incorporated into *M. leprae* DNA.

Two kinds of culture media were selected. The first one is DH medium in which Dhople and Hanks had successfully achieved growth and subcultures of *M. lepraemurium*, and the second one is Mahadevan's conditioned medium using supernates of dorsal root ganglian cultures. The cultures containing *M. leprae* in these two media were incubated at 34°C. After an initial lag of 4–6 weeks, there was a definite multiplication of *M. leprae* in both the media. The maximum growth, as judged by all three of the above criteria, was obtained between 14 and 16 weeks. Even though the rate of multiplication was slow and the cell yield was very low, the harvested cells were shown to be *M. leprae* by several standard tests. The cells harvested from both the culture media after 16 weeks were used to inoculated freshly prepared respective media and the cultures were incubated at 34°C. During the 12 weeks of incubation there was a steady and constant decline of bacterial ATP, DNA and also (3H) thymidine uptake suggesting that metabolically the cells became totally inactive. The cells recovered at the end of 12 weeks failed to multiply in the foot pads of mice. Thus, it can be stated that there was a limited but definite multiplication of *M. leprae* in primary cultures but subcultures could not be achieved. Since then several modifications have been made in both the culture media to improve growth rates as well as cell yields.

Next, DH medium was employed to evaluate the effects of DDS and rifampicin. *M. leprae* were incubated in the presence of various concentrations of DDS and at periodic intervals, the cells were taken for ATP assays and (3H) thymidine uptake. No inhibitory effects were seen when the concentration of DDS was 10 ng/ml or less. At the end of 6 weeks, *M. leprae* became non-viable in the presence of 20 ng/ml DDS and this period decreased with the increasing concentration of DDS in the medium. *M. leprae* harvested at the end of 8 weeks of incubation were inoculated into the foot pads of mice to compare above *in vitro* results on viability. Similarly, using this method the MIC of rifampicin against *M. leprae* was found to be between 250 and 300 ng/ml. These studies are in progress.

19 Single bacterial cell mass analysis: a rapid test method in leprosy therapy control. U Seydel & B Lindner

To overcome problems arising from the *in vitro* non-cultivability of *M. leprae* we have started some time ago to develop an alternative technique to acquire fast and reliable information on the effectiveness of a chemotherapy by mass spectrometric analysis of a single *M. leprae* cells isolated from biopsies.[1,2] The information is derived from measurements of the intracellular concentrations of sodium and potassium ions and from the evaluation of so-called mass fingerprints which stem from fragment ions of the complex cell matrix.[3] All information is available within hours after the preparation of the samples from biopsies.

So far, it could be shown that the ratio of the intracellular sodium and potassium ion concentrations (Na^+, K^+-ratio) is a sensitive indicator of the physiological state of a cell and that its value can be taken as a measure for the impairment of a cell following chemotherapy. From first evaluations of the time dependence of the Na^+, K^+-ratio in a follow-up study it may be expected that the method can yield information on kinetics of drug interaction. The data extracted from mass fingerprint evaluation, furthermore, gives evidence for its applicability for monitoring the development of drug resistance.

In close cooperation with Dr A M Dhople (Melbourne, Florida/USA) a good agreement between the statements for his ATP-assay, the mouse footpad test and our measurements of the Na^+, K^+-ratio was found.[2]

A particular advantage of the single cell mass spectrometry, however, is—beside the fact that all data are obtained from the analysis of only a few hundred cells—the possibility to get more detailed insight in the drug response of a cell population by analysing single cells and this way getting distributions of the respective data instead of averaged values.

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20 Metabolism in *M. leprae:* possible targets for drug metabolism. P R Wheeler

Metabolic activities in *M. leprae* which are essential for the growth and survival of the bacteria, and not present—or present but with completely different properties—in the host, are potential targets for anti-leprosy agents.

Much is known about the energy metabolism in *M. leprae*, including the dissimilation of carbon sources. However, most of the pathways are widely distributed amongst living organisms, and there often exist 'alternative pathways'. Thus energy metabolism may not be amenable to inhibition by anti-leprosy agents although two activites in *M. leprae*—glycosidases possibly involved in Hexuronate catabolism, and cytochrome o—are characteristically bacterial and specific inhibitors may be found there.

Generally, antibacterial drugs act against biosynthetic activities or replication (which can be seen as the culmination of many, co-ordinated biosynthetic activities in the bacterial cell). Studies of biosynthetic activities in *M. leprae* are fragmentary, but synthesis of the cell-wall can be discussed. In most respects, the wall of *M. leprae* is similar to that of other mycobacteria, so any agents developed which act on the cell wall of other mycobacteria should inhibit growth of *M. leprae*. Protein synthesis in *M. leprae* is characteristically bacterial, being inhibited by chloramphenicol. One amino acid not incorporated into protein is DOPA, yet this amino acid, as either L-DOPA or D-DOPA, is taken up and oxidized by *M. leprae*, interestingly an activity which appears restricted amongst the mycobacteria to *M. leprae*. The biological significance of DOPA oxidation is not known and it may be that in attempting to design agent against DOPA 'metabolism' an activity of no importance to the bacteria is being selected as a possible target.

Nucleic acid synthesis the target of many antibacterial drugs, and two agents effective against *M. leprae*, rifampicin and clofazimine, appear to affect these pathways. Thiosemicarbazones appear to inhibit mycobacterial ribonucleotide reductase, an enzyme for making nucleotides available for DNA synthesis. Much is known about the synthesis of nucleotides by *M. leprae*: it is doubtful whether de novo synthesis occurs in *M. leprae*, but the organisms scavenge purines very effectively. If *M. leprae* proves to require purines for growth, then there exists the possibility that drugs could be developed against purine scavenging in *M. leprae*. Indeed, pyrazolopyrimidines are known to inhibit growth of some pathogenic trypanosomidae which are dependent on preformed purines. The use of hyposanthine incorporation as a potential, general drug screening method will be briefly discussed.

Although the development of agents against folate metabolism will be discussed elsewhere, I will discuss the metabolic significance of inhibiting folate metabolism briefly in this talk.

21 Host-pathogen interaction—new *in vitro* drug test systems against *M. leprae*—their possibilities and limitations. P R Mahadevan

Mycobacterium leprae which has so far failed to grow even slowly *in vitro* or even metabolize actively on *in vitro* isolation, poses a problem for rapid drug sensitivity assay. The only drug test system that was possible till recently is using the growth potential of this bacterium in mouse footpad—an assay that would take at least 9 months to show drug sensitivity or resistance of *M. leprae* to the test compound. To overcome the disadvantages mentioned above, we have directed our attention to *M. leprae*-induced changes in host cells, as part of host-pathogen interaction. Having identified such changes it was possible to monitor such changes in presence or absence of drugs which would indicate inactivation or confirmed viability of *M. leprae* respectively. The indicator changes were involved in host cell membrane receptors, protein synthesis and activation of *M. leprae* metabolism.

Exploiting the above criteria, we have developed few *in vitro* assay systems—three of them are referred to as (a) Fc receptor assay, (b) FDA-EB assay and (c) Uracil uptake assay. There are others with potential use; they also will be described.

All these assay systems basically use cultured peritoneal macrophages from mice, exposed to the test drug in presence of phagocytosed *M. leprae*. The expected changes when live bacilli are present are monitored. If such changes do not occur in presence of a drug, the drug is considered as active.

Using all the three assay systems and some others, susceptibility of *M. leprae* to sulphone and rifampicin has been demonstrated and loss of viability of *M. leprae* in such experiments were also correlated with mouse foot pad tests. This correlation showed the validity of these test systems.

Some new compounds have been identified as potential anti-*M. leprae* agents by the above *in vitro* assay systems. One such compound is Brodimoprim. Its synergistic activity with dapsone against *M. leprae* was demonstrated and this has been confirmed in mouse foot pad.

Other drugs identified are Deoxyfructoserotonin, ciprofloxacin, Indole-2-carboxylic acid, Diflunisal and few other derivatives from the laboratories of Dr J K Seydel.

Two of the test systems were subjective, since it involved use of microscope by the investigator and counting. But both of these have been now confirmed by a more quantitative method. Fc receptor assay is demonstrated using I^{125} labelled antibody coated SRBC. FDA-EB method by measuring fluorescence by spectrofluorimeter.

The advantages of these *in vitro* assay systems are (a) it is completed in less than 10 days, (b) in vitro MIC can be determined, (c) synergestic activity between two different drugs can also be established, (d) static or cidal effect can be assessed. Among the drawbacks (a) one needs at least 5–10 million *M. leprae* for each assay, as compared to 1×10^4 in mice footpad. (b) As patients improve on drug therapy viability goes down thus to monitor viability one has to use higher number of bacilli and this may lead to ambiguous data.

However, we are in a better position now to identify potential anti-*M. leprae* compound much faster than we were 5 years ago. Information regarding the above assay systems, observations and conclusions will be presented and discussed.

22 Isolation of environment-derived *M. leprae* and its application in cultivation trials. J Kazda

It is well known that even in highly endemic areas, contact with leprosy patients cannot be established as a source of infection in a considerable proportion of new cases. In a study covering Africa, Asia and the United States, this contact could be established in 25 to 60%. Already at the IInd International Leprosy Congress in 1909 SAND postulated on the basis of epidemiological

observation in Norway, that leprosy is not transmitted by direct contact, but probably through some environmental medium, such as soil.

In samples collected in Bombay Leprosy Area an isolation of environment-derived *M. leprae* has been described recently. This strain of *M. leprae* was isolated from soil in foot-pad technique in mice. Besides biochemical properties specific for *M. leprae* (dopa oxidase and pyridine decolorization), the specific phenolic glycolipid I could be detected.

Using a direct inoculation of a suspension of the same soil sample in sphagnum nutritive substrate, used for cultivation trials with *M. leprae*, an additional mycobacterium, grown on conventional media, has been found together with *M. leprae*. This mycobacterium, identified as *M. intracellulare*, serotype Darden, caused an increase of pathogenity of *M. leprae*, when inoculated in foot pads of nude mice. The swelling of foot pads, observed 4 to 6 months after inoculation, was accompanied with the development of cutaneous leproma in the dorsal site of the nude mice.

The first experiments with the influence of *M. intracellulare*, serotype Darden on the multiplication of *M. leprae in vitro* are described.

23 Investigations into the cultivation of *M. leprae*. A multifactorial approach. L Kato

Culture media for *M. leprae* are proposed on the assumption that the mycobactin deficient *M. leprae* requires growth factors produced by leprosy-derived mycobacteria (LDM). *M. intracellulare* and *M. phlei*—both LDM—were used as donors of exochelins and/or mycobactins in the multifactorial media. The LDM—*M. phlei* and *M. intracellulare* were grown respectively for 10 and 20 days in Sauton medium or a basal medium without and with Tween 80 added. Autoclaved cultures were filtered and Na thioglycolate 1 g, thioctic acid 0·1 g, $(NH_4)\ SO_4$ 2 g, $MgSO_4$ 0·1 g and ferric ammonium citrate 0·05 g were dissolved in 1 litre of the filtrates. The pH was adjusted to 5·8 with KH_2PO_4. Twenty ml media was distributed into each of 25 ml screw-cap tubes and autoclaved for 30 minutes. The media thus prepared contained sufficient amounts of exochelins and mycobactins to support growth of *M. paratuberculosis* ATCC 19698.

Host-grown *M. leprae* were inoculated into the multifactorial media and incubated at 34°C. Positive growth and subcultures were obtained from 3 out of 4 specimens in the exochelin-mycobactin enriched media.

Latency period of growth was estimated at 10–16 days and time of division at 8–12 days. Cells were long, acid-fast, arranged side by side or end to end, with a tendency to form long spiral cords or clumps when sedimented on siliconized slides. Pyridine extraction eliminated acid fastness, but not Gram positivity. Cultures did not grow on Dubos, Löwenstein or 7H10 media. In the footpads of mice they produce the disease characteristic of *M. leprae*. Subcultures remain dependent on the multi-factorial media, enriched with growth factors (exochelins and mycobactins) from the leprosy-derived cultivable mycobacteria.

SESSION III. DEVELOPMENTS AND FUTURE ASPECTS. *CHAIRMAN:* ENNO FREERKSEN (FRG)

24 Recent developments in the field of multidrug therapy and future research in chemotherapy of leprosy. J H Grosset

The discovery of rifampicin together with the increasing prevalence of dapsone resistance were decisive factors to question the value of dapsone monotherapy and even of any drug monotherapy

in the treatment of leprosy. Thanks to the efforts of some leading personalities and of WHO a progressive move took place during the decade 1970–80 towards a multidrug therapy of leprosy as that was the case in the therapy of tuberculosis since the early fifties.

Besides to stop the transmission of the bacilli in the coummunity, chemotherapy for leprosy as well as for tuberculosis has two objectives: (i) to prevent the selection of drug resistant mutants and (ii) to kill the drug sensitive organisms. To reach the first objective, a combination of drugs active against *M. leprae* should be given as long as the drug resistant mutants present at the beginning of treatment have not been eliminated. To reach the second objective one single sterilizing drug or a combination of sterilizing drugs should be given for a length of time sufficient to prevent the majority of relapses due to the regrowth of persisting organisms.

Although the more recent controlled clinical trials and field trials conducted in different parts of the world have demonstrated the high effectiveness of multidrug therapy, the precise length of time necessary to eliminate all drug resistant mutants is not yet known. This is therefore one of the priority of the research in chemotherapy of leprosy. While WHO and other organizations are working to solve the problem, it is necessary to use a three-drug combination throughout the whole course of chemotherapy for multibacillary leprosy, as recommended by WHO.

Another problem to be solved is the precise length of treatment necessary to prevent relapse after stopping treatment. Is the 2-year treatment too long or too short for all cases of multibacillary leprosy? What is the relationship between the presence and the number of persisters and the risk of relapse after stopping treatment? What is the effect of extending chemotherapy beyond the minimal course of 2 years on the number of persisters and on the relapse rate? What is the role of immunotherapy about that? These questions should be and will be answered in the future by research programmes. Meanwhile it is safe to follow the WHO recommendations that is to treat multibacillary patients for a minimum of 2 years or at best till the negativation of BI.

25 Strategies in the development of new drugs and drug combinations against leprosy demonstrated on the example of folate- and gyrase-inhibitors. J K Seydel, M Rosenfeld, M Sathish, R. Haller, M Kansy & G Hachtel

The lack of an *in vitro* test system for *M. leprosy* is forcing us to think about new routes for the development and screening of potential antileprotic drugs and drug combinations. This is relevant for testing known antibacterials produced by pharmaceutical companies as well as for the development of new drugs against leprosy.

Mouse foot pad technique—besides being very time consuming—is only of limited value because of decisive differences in pharmacokinetics and metabolism of drugs in mice and man. This can lead to underestimation of the effectivity of the drug in mice (false negatives). In addition the observed effective dose is not relevant for the dose necessary for cure in man. These problems are discussed on the example of new quinolonic acid derivatives (Ciprofloxacin®, Ofloxacin®) and for new folate synthesis inhibitors developed in our laboratories. Test systems used are cell-free enzymes, cultivable mycobacterial strain, *M. leprae* suspensions (Dhople) and serum activity tests in human.

The new folate inhibitors are up to 300 times more effective against mycobacteria as compared to known folate inhibitors (trimethoprim, pyrimethamin). A strong synergism in combination with dapsone is observed.

According to the obtained results quinoline acid derivatives and combinations of the new inhibitors of bacterial folate synthesis are promising compounds for the treatment of leprosy.

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26 Development of inhibitors of mycobacterial ribonucleotide reductase. K-J Schaper, J K Seydel, M Rosenfeld, J Kazda & H Schönenberger

For several reasons there is an urgent need for new drugs for the chemotherapy of leprosy. Starting with the known but unsatisfactory activity of thiacetazone against *M. leprae* or the leprosy model strain '*M. lufu*', a screening of related thiosemicarbazones (TSCs) showed that 2-acylpyridine-TSCs are considerably more active against '*M. lufu*' than other TSCs lacking the basic N atom in the alpha-position. Literature results suggest that these metal ion chelators are acting as inhibitors of the iron containing bacterial enzyme ribonucleotide reductase.

The toxicity of acylpyridine-TSCs was considerably reduced by replacing their thioamide group by different N-heterocycles (new lead PH22). This exchange furthermore caused an increase of both antibacterial activity and chelating properties.

In accordance with the mode of action hypothesis of ribonucleotide reductase inhibition it was found in cell cultures that PH22 derivatives are very potent inhibitors of DNA synthesis. Interestingly a very pronounced synergism in antimycobacterial activity is observed on combination of PH22 with several drugs known to be inhibitors of the DNA synthesis pathway.

27 Successful regimens of definite duration in the treatment of leprosy. Theoretical considerations and practical results. S R Pattyn

As we pointed out in the past the outlook of leprosy treatment was revolutionized by the discovery of the bactericidal activity of rifampicin (RMP) and the thioamides (THA). Based on theoretical considerations and results of experimental chemotherapy in the mouse, paucibacillary leprosy should be curable by a relatively short course regimen with a bactericidal drug in monotherapy. Multibacillary leprosy should be treated by combined therapy. Questions to be answered are: (a) what combination(s) of drugs, (b) at what frequency or intermittency should drugs be administered, (c) how long should treatment be persued.

Precise definitions and criteria for cure have to be defined, the most important being the killing of all bacilli and the occurrence of absence of relapses, equally to be defined precisely.

The ideal treatment regimen is the one dose therapy, the 'therapia sterilisans magna'. Since this cannot be realized yet with the drugs available, treatment regimens approaching this goal have to be defined. Regimens should be efficaceous and therefore supervisable and for this reason of short duration, eventually intermittent.

Since circumstances are largely different in the world, there is need for different regimens with known efficacy, to allow Public Health Authorities to make a rational choice of drug regimens that suit their possibilities at best.

The only method to measure the value of drug regimens is the conduction of prospective, eventually comparative, clinical trials, With this in mind we conducted over a number of years several prospective studies on the efficacy of different drug regimens in various forms of leprosy.

In PB leprosy we have studied the following regimens, the results of which will be illustrated and discussed.

1. RMP 900 mg 1/7 8×.
2. RMP 900 mg 1/7 10×.
3. RMP 900 mg 1/7 12×.
4. RMP 600 mg 1/7 10×.
5. RMP 600 mg 1/30 6× (WHO regimen).

In MB leprosy we have studied regimens of:

1 year duration:
6 M RMP 600 7/7 ETH 500 7/7 DDS 7/7 + 6M DDS 100 7/7 with or without ETH 500 7/7.

of 6 months duration:
2W RMP 600 7/7 ETH 500 7/7 DDS 100 7/7 + 24W RMP 600 1/7 ETH 500 7/7 DDS 100 7/7.

of 3 months duration:
RMP 600 2/7 ETH 500 7/7 DDS 100 7/7.

The results will be illustrated and discussed.

28 Preliminary results of treatment of leprosy in the Netherlands with an alternative drug combination. D L Leiker

SESSION I. CLINICAL ASPECTS

Chairman: D L LEIKER (THE NETHERLANDS)

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The Malta experience. Isoprodian–rifampicin combination treatment for leprosy

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In June 1972, the Malta Leprosy Eradication Programme was started with the cooperation of the Malta Government, the financial support of the German Leprosy Relief Association and the Sovereign Military Order of the Knights of Malta, and the assistance of the Borstel Research Institute.

The basic concept was to treat all leprosy patients known at the time, as well as any subsequent newly diagnosed patients, with an antimycobacterial combination of high effectivity, for a limited period of time as determined by their individual clinical and bacteriological progress. All patients were then to be kept under regular post-treatment control for the possibility of relapse. From *in vitro* studies, as well as clinical trials previously carried out in other countries by Freerksen and co-workers, it had been established that a fixed combination was imperative.[1,3,4] Besides being therapeutically effective, this combination had to be administered orally and be well tolerated so as to ensure high patient acceptability and compliance. The combination Isoprodian–rifampicin was found to fit these criteria and was thus employed in the programme[2].

Since the therapy was administered entirely orally, the programme could be carried out on an outpatient basis from the Dermatology Department of the only general hospital on the island. With this arrangement, patients could be referred to the outpatients of any department with little inconvenience to the patient. If and when necessary, they could also be admitted to the general wards of the hospital as inpatients. In order to facilitate matters for the patients and ensure further compliance, a fortnightly outpatient clinic was also held on the sister island, Gozo. In cases where patients were unable to attend the outpatient clinics for some reason, home visits were regularly carried out.

During the treatment-period patients were examined at weekly or fortnightly intervals for the initial two or three months and then on a monthly basis. Skin smears and a biopsy were taken from every patient monthly, and these were examined at the Borstel Research Institute, where homogenated as well as histological sections were performed on all biopsy material. When the necessity arose, fresh biopsy specimens were also sent to Borstel for mouse footpad

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innoculation. Skin smears were also examined in Malta for reference. During the post-treatment control period, patients were initially seen on a monthly basis and then at progressively longer intervals of two, three and six months. Smears and a biopsy were taken at every visit.

Since the start of the programme a total of 247 patients have participated. Of these, 201 were registered patients known to be suffering from leprosy at the onset of the programme, and until then, had been treated with dapsone monotherapy for a variable number of years. Forty-six newly-diagnosed patients have been included in the programme since 1972.

Figure 1 shows the age and sex distribution of the patients at the time of starting multiple drug therapy (MDT). There were no patients below the age of ten, the youngest patient being a boy aged 11, while the oldest was a man of 81 years.

Figure 2 shows the patients grouped according to the type of their disease. In a number of patients with long-standing disease, precise classification was not always possible, and therefore patients with LL and BL are grouped together as lepromatous, while those with TT and BT are likewise grouped as tuberculoid. It is clear from the figure however, that the large majority of the patients (77%) were suffering from lepromatous leprosy.

Irrespective of the type of leprosy and any previous treatment, all patients were treated with the same combination of rifampicin (2 capsules × 300 mg) and Isoprodian (2 tablets) daily for 6 days per week. Isoprodian is a fixed

Years	M	F	T
0 - 9	-	-	-
10 - 19	7	2	9
20 - 29	5	13	18
30 - 39	21	17	38
40 - 49	41	26	67
50 - 59	29	26	55
60 - 69	36	9	45
70 +	8	7	15
	147	100	247

Age and Sex Distribution

combination, each tablet containing 75mg of isoniazid, 75mg of prothionomide, and 50mg of dapsone. Patients under the age of 16 years and adults whose body-weight was below 45 kg were given half the above-mentioned dose. More recently, it has been possible to give the required dosage of all four drugs in a fixed combination: Isoprodian–RMP. With this further development, administration is easier, thus ensuring greater patient compliance. It also avoids the possible risks of rifampicin monotherapy or blackmarketing.

Out of the 247 patients that started MDT, four patients were excluded from the programme for the following reasons. Two patients were found to be unreliable and were not taking the treatment regularly, if at all; one patient refused further treatment after 6 weeks due to persistant vertigo, while the fourth patient refused further treatment following a lepra reaction 2 weeks after starting MDT.

In conformity with the predetermined plan of the eradication programme, patients who had been found to be bacteriologically negative at the start of the programme were treated with MDT for a fixed period of 5 months. In all other cases the time of termination of therapy was determined by the clinical and bacteriological progress of the individual patient. Figure 3 shows the duration of MDT of the patients included in the programme, except for three patients who have started treatment in the last two years and are still undergoing therapy.

One hundred and four patients did not receive rifampicin throughout the whole of their treatment period, but for the first 5–10 months after which treatment was continued with Isoprodian alone for varying periods of 6–15 months. This was not done due to any adverse reaction to rifampicin, but because the clinical and bacteriological situation of these patients was sufficiently satisfactory to warrant the withholding of rifampicin from the treatment regimen with a considerable reduction in cost. (In a minor variation from the original protocol, the usual Isoprodian–rifampicin combination was replaced by the combination rifampicin (600 mg) prothionomide (300 mg) and ethambutol (1200 mg) daily for a few months in a group of 15 patients. However, since no

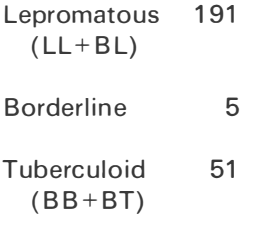


Figure 2. Classification of patients.

notable superior effect was recorded, the original combination was again administered.)

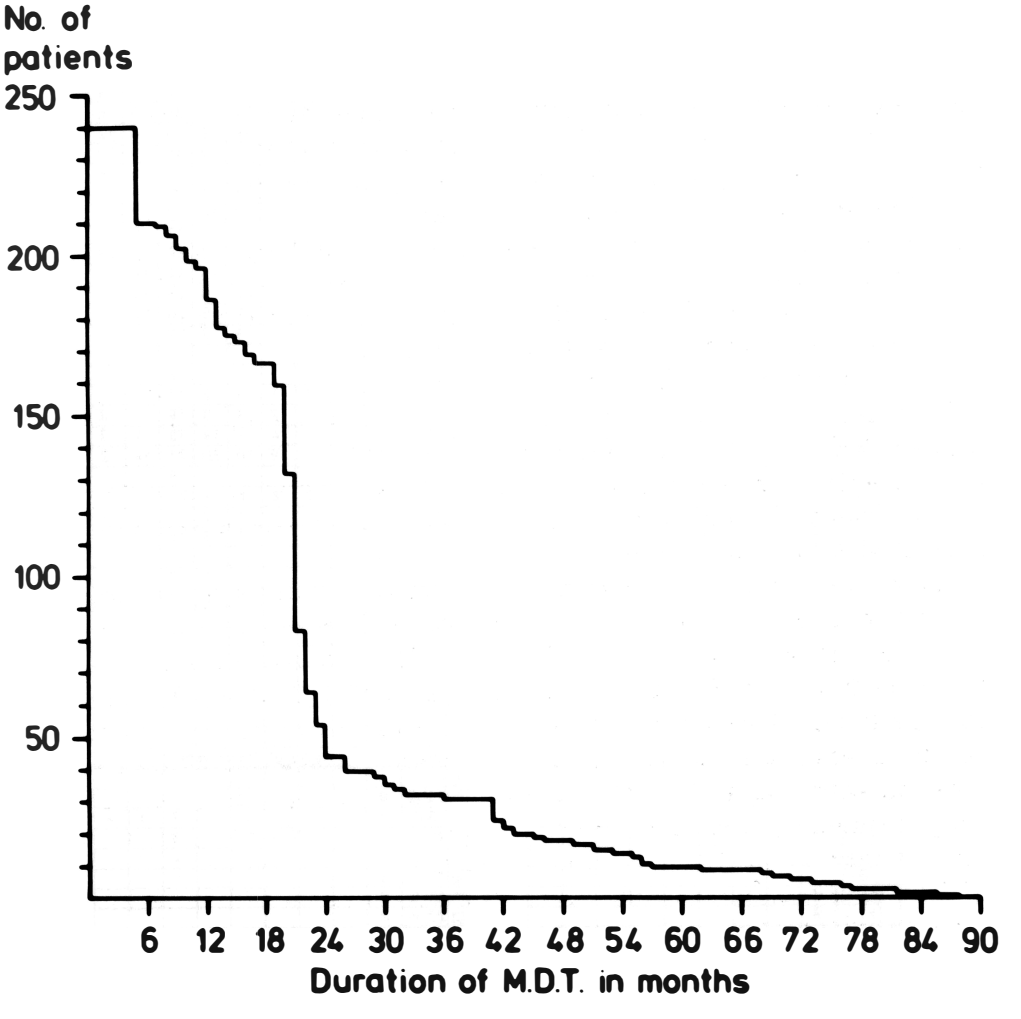
As mentioned earlier smears and biopsies were sent to Borstel regularly. Smears were flame-fixed in our clinic in Malta and sent to the reference laboratory at Borstel for staining and evaluation. The results were recorded according to the scheme shown in Figure 4. Here we see a typical course of bacteriological regression. In our opinion it is very important to get a continuous picture by taking smears monthly, and not by singular findings at long intervals. Figure 4 shows the course in a classical simple case. The analysis shows that all forms of bacteria disappear quickly, especially the 'solid' and 'fragmented' forms, while the forms listed in columns IV–VI take longer to disappear. The total regression and the concentration of these forms are very typical.

As can be seen bacteria may still be found at various times after the withdrawal of treatment. However, this is of no significance to the overall course. Finally, bacteria can no longer be detected. In the case in question, bacteria were no longer found after mid-1980. This signifies that one need not wait until the BI has reached zero to stop treatment.

The overall course of bacterial regression always follows this principle, despite all differences seen in the individual cases. The next case (Figure 5) was treated for almost three and a half years. You can see the same principle underlying the course of bacterial decline, but a longer period of time was needed until a continuous bacterial regression became evident. A relatively large number of bacteria was still to be seen for a longer period of time after withdrawal of treatment, with intermittent periods during which no bacteria were detected during the monthly check-ups. As can be clearly seen, this case could be released from treatment despite the detection of bacteria. Finally, the BI reached zero.

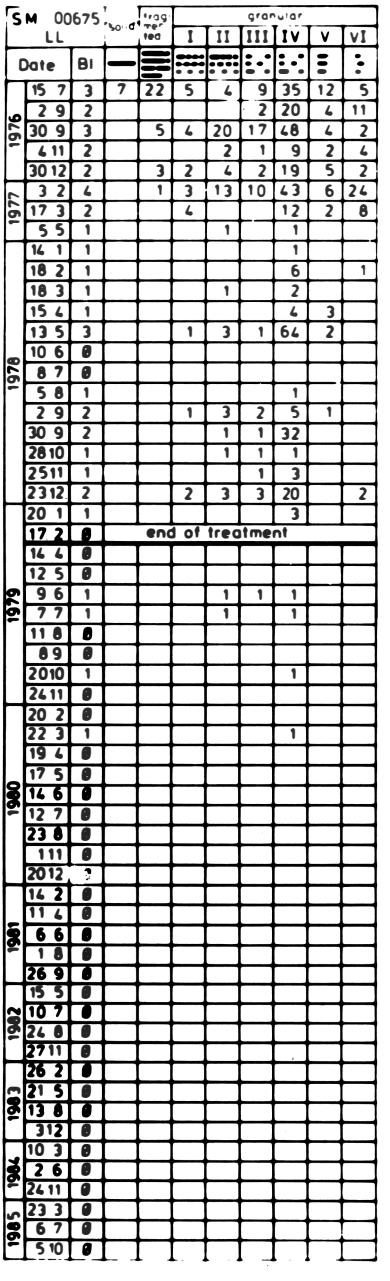
The recognition of the course of bacterial regression and its correlation with the clinical picture give a clear indication for the timing of release from treatment.

From a clinical point of view, the most dramatic changes in patients undergoing treatment with Isoprodian–rifampicin combination was the rapid healing of ulcerating nodules and the early regression of infiltrated lesions in lepromatous patients. Nodules and diffuse infiltration of relatively recent origin subsided in a matter of a few weeks to a few months. This was also observed in a number of patients with long-standing fibrotic lesions that were not expected to resolve to any significant degree at the start of MDT. Similar changes were recorded in patients who had signs and symptoms of mucosal involvement at the start of MDT. Nasal symptoms were relieved in the first weeks of treatment, while in three patients initially suffering from hoarseness due to laryngeal infiltration, the voice returned to normal in a few weeks. The unexpected regrowth of eyebrows in a few of the patients with madarosis was very comforting to them. This occurred mainly in those patients where the thinning or loss had first been noticed during the last two years prior to the initiation of MDT. A considerable number of patients with sensory impairment reported improvement in their



sensory ability. However, on neurological examination clinical improvement in sensation could only be recorded in a small number of cases.

Prior to the use of MDT the occurrence of Type 2 lepra reaction in patients under treatment was frequent and often severe. This is hardly surprising considering the fact that the higher proportion of leprosy patients in Malta belong to the lepromatous side of the spectrum. It had often been the practice to withhold or lower the dosage of dapsone during reactions. It was decided at the outset of the programme that no alteration in dosage should be made in the case of lepra reaction. The performance of patients with regard to lepra reaction was consequently given all due attention.



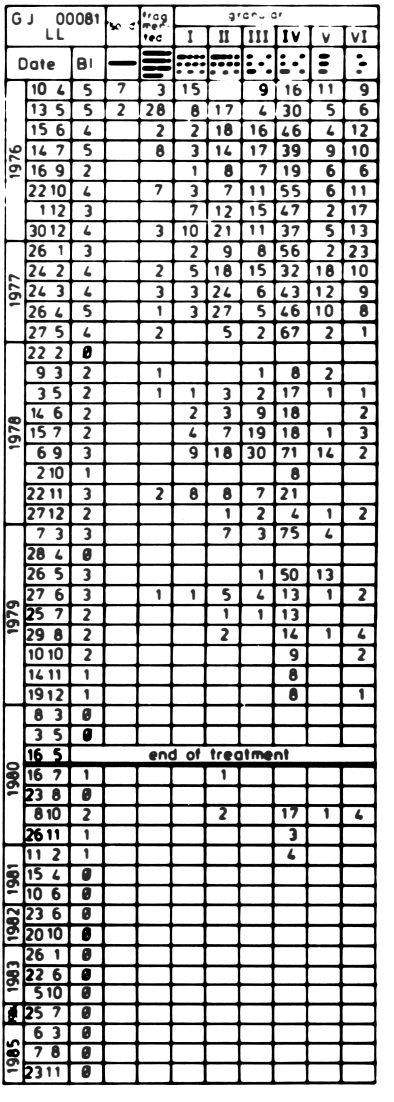


Figure 4. Serial bacteriological results of a typical LL case during the treatment and control periods. Bar denotes time of stopping MDT.

Figure 5. Serial bacteriological results of another LL case showing a longer period of MDT.

Of the 243 patients treated with MDT, 10 had Type 1 lepra reaction, while 86 patients had one or several episodes of Type 2 lepra reaction. A considerable number of these reactions were mild, with just crops of evanescent nodules and no constitutional symptoms. Forty-two patients had one moderately severe reaction with constitutional symptoms such as: pyrexia, joint pains, myositis, etc. Two cases were severe enough to warrant hospitalization for a few days. Erythema necroticans was, however, not encountered. In 37 patients, episodes of mild reaction were also recorded during the first 2 years after stopping treatment.

Most reactions were satisfactorily controlled by the administration of 100–400 mg of thalidomide at night, dosage being varied according to severity. Corticosteroids were used in as small a number of cases as possible in view of the high incidence of diabetes in Malta.

Ocular involvement was a reasonably frequent occurrence prior to MDT, as indicated by the ophthalmic examination of the 201 patients who were admitted into the programme in June 1972. Eight of these patients had blindness of one or both eyes, while a further 23 patients had less severe involvement, such as, diminished vision, corneal opacity, sclerosing keratitis, and irregular or non-reactive pupils. During the course of our study, five patients had an episode of irido-cyclitis, all of which responded satisfactorily to treatment. It is gratifying to note that no case of blindness has ensued in the group of 46 patients diagnosed since 1972 and treated with MDT.

Out of the 116 male patients who started the programme in 1972, seven gave a past history of one or more episodes of epididymo-orchitis. However, no episode was recorded subsequent to the start of MDT in these patients or in any of the other 31 male patients who were included in the programme since 1972.

Most patients tolerated the Isoprodian–rifampicin combination without any difficulty. The commonest side-effects encountered were gastrointestinal disturbances. These included: nausea (64 cases), dyspepsia (23), vomiting (19) and anorexia (5). These side-effects were mainly reported in the first 2 or 3 weeks and abated spontaneously on further treatment in most cases. Eight patients, however, continued to suffer from occasional dyspepsia and four patients reported episodes of nausea from time to time throughout the treatment period. Other side-effects encountered in the first 4 to 6 weeks of treatment were: soreness of the mouth and tongue (28 cases), dizziness (17), and lassitude (5). An acneiform rash developed in three patients, while two patients complained of paraesthesiae. Two patients developed depressive psychosis which could have been contributed to by the treatment.

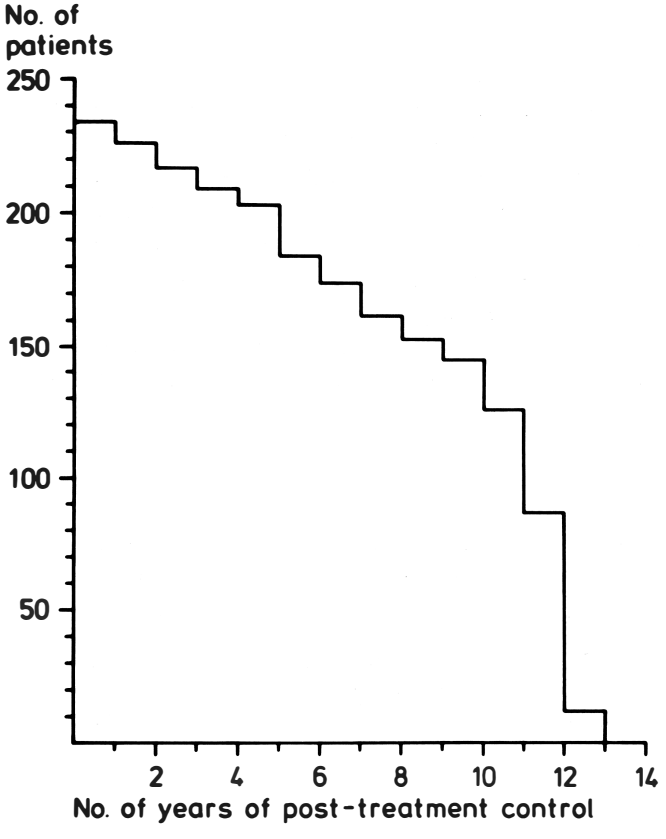
Twelve patients had moderate anaemia, while a further 21 patients had mild anaemia in the course of treatment. However, it was observed that these episodes of anaemia occurred mainly during lepra reactions. White blood counts and platelet counts were within normal limits.

Liver function tests were not routinely performed prior to 1978. Up to that time, a rise in serum bilirubin and/or serum transaminases was recorded in eight

patients. These rises were of a low grade and results returned to normal without the interruption of therapy. Two other cases developed jaundice and necessitated the suspension of antileprosy treatment until liver function tests returned to normal. When treatment was restarted the serum transaminases of one patient showed progressively increasing levels necessitating interruption of therapy, while the other patient completed her course of treatment uneventfully.

Since 1978, 18 patients have had routine fortnightly or monthly liver function tests performed during the whole of their course of treatment. Ten patients had normal LFTs throughout. Six patients showed transient elevations of serum bilirubin and/or transaminases, the levels of which returned to normal despite continuation of treatment. Two patients had progressively increasing levels of serum transaminases necessitating interruption of treatment. These settled to normal but started rising again when treatment was restarted.

There can be no doubt that in a programme like that undertaken in Malta, the aspect of post-treatment control is of the utmost importance. The incidence of relapses during this control period can be considered as a gauge of the efficacy of



the treatment regimen employed. One would expect that the majority of patients, where treatment has been ineffective, would show signs of relapse in the first 2 or 3 years following cessation of treatment. The situation in Malta from the point of view of control was ideal in that the size of the country greatly facilitated patient surveillance, thus allowing post-treatment control for a much longer period. In fact, apart from deaths due to natural causes, regular control of all 240 patients who have completed their treatment has been carried out, with the exception of only seven. Four patients have emigrated from Malta at some time during the control period, while three patients have refused to attend for further follow-up after a few years.

Figure 6 shows the duration of control for all patients who have completed their course of MDT. As can be seen from the diagram, 145 patients have been under regular control for 10 years or more, while a further 57 patients have been controlled for a minimum of 5 years. No clinical relapse was registered in any of the patients under control. In one female patient who had received MDT for a period of 5 months, a routine biopsy in the 6th year of control indicated the possibility of a bacteriological relapse. Although there was no clinical evidence of any relapse, it was considered prudent to restart treatment. The patient was given a second course of Isoprodian–rifampicin combination for 18 months and has since then been under control for over 5 years without any evidence of relapse.

I would here like to express our appreciation of the extreme cooperation we have had from the patients with regard to their regular attendance and compliance during both the treatment and control periods.

In conclusion, it may be stated that all 243 patients were treated with the same Isprodian–rifampicin combination which could be administered easily and which was well tolerated. The duration of therapy was determined by individual clinical and bacteriological progress. After treatment all patients were kept under regular control and practically no relapses were recorded.

It is of the utmost importance that the implications of the results achieved in the Malta programme are followed up by on-going observation as is presently carried out.

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On the epidemiology of leprosy in Malta

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The Malta Project was not primarily set up as a trial of the combination of rifampicin–Isoprodian, but as a leprosy eradication project. Therefore the question that should be raised is whether or not Malta is a suitable area for demonstrating eradication of the disease by mass-chemotherapy. In order to answer this question epidemological data are required, in particular about the trend of the disease prior to the introduction of MDT. Unfortunately, data based on population surveys are not available. However, it was possible to recover all notifications of leprosy to the Ministry of Health since 1900. Notifications do not necessarily reflect the incidence of the disease. In Malta, being a relatively small and close community where few patients escape notice when the disease becomes more advanced, it is believed that, although the notifications do not give a complete picture of the incidence of the disease, they give valid information on the trend of the disease. After screening of the records for duplication a clear and consistent pattern emerged.

The curve of notifications shows two peaks, corresponding with the opening of a male hospital in 1900 and the opening of a female ward in 1911. The number of notifications between 1915 and 1965 remained rather steady; after 1965 the number of notifications decreased significantly. Sulphone therapy was introduced in Malta in 1955.

At first glance the number of notifications suggest that the decrease started only about 10 years after the introduction of chemotherapy. However, if the numbers are related to the population figures a different picture emerges.

The number of notifications per 10,000 population (based on census figures) have already shown a steady decrease since 1900, long before chemotherapy was introduced and there is no evidence of a marked additional effect of chemotherapy.

In the first decades of the century there is no marked shift in the average age of notification towards higher age groups. In the last 20 years there is a shift towards

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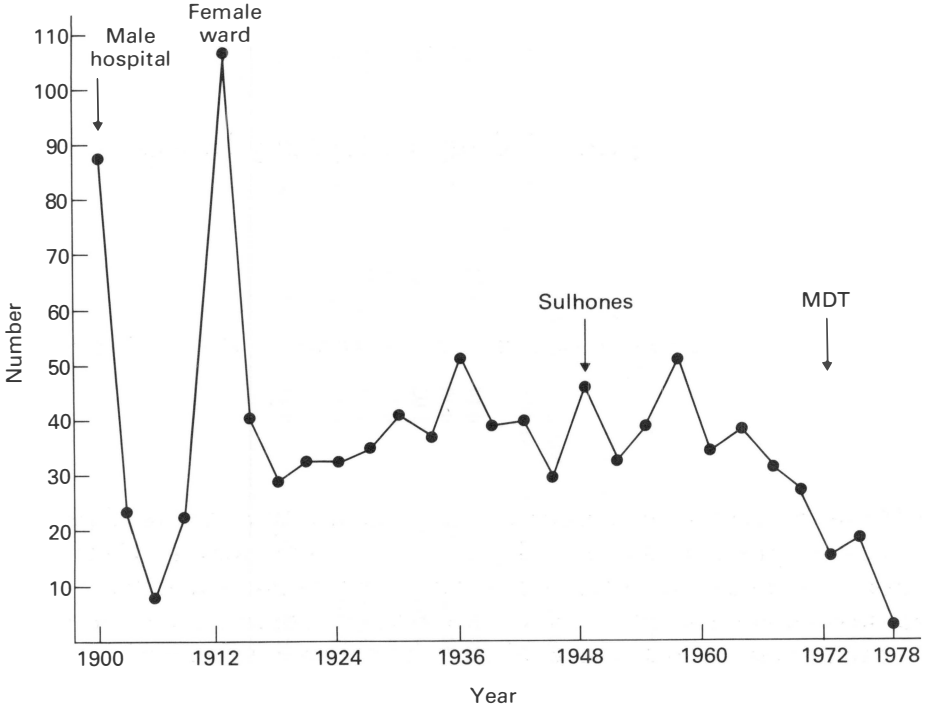

Figure 1. Notifications of leprosy to the Ministry of Health.

a higher average age, which is in support of a decrease in transmission of the disease.

In a proportion of the patients notified in the last decades it was possible to identify the most probable type of contact with a source of infection.

Table 2 shows that in two-thirds of the patients a contact in the family could be identified. The age of onset of the disease in patients with family contact is, on average, significantly lower than that of patients without family contact. The average age of onset of patients with a mother suffering from leprosy was lower than that of patients whose father or another relative was a patient. In Malta the disease shows marked clustering in families, which is compatible with a relatively low rate of transmission. When the figures are studied in greater detail significant differences are found between various parts of the island (Table 1).

In the capital Valetta, the incidence of leprosy was already low in 1900 and remained at the same level, in spite of the fact that large numbers of people from more highly endemic rural areas have moved to the capital, either permanently or as day-time labourers. Apparently the conditions for transmission of the disease in the capital were less favourable than in the rural areas.

Already in the first decades of the century a very marked decline of the disease is reflected in the figures of the Outer Harbour area. This corresponds with a rapid

Table 1. Notifications per 10,000 population per year in the districts.

	1900–1919			1920–1939			1940–1959			1960–1978		
	Census 1911	No. notifications	Notifications/ yr/10,000	Census 1931	No. notifications	Notifications/ yr/10,000	Census 1948	No. notifications	Notifications/ yr/10,000	Census 1967	No. notifications	Notifications/ yr/10,000
Inner Harbour	93,783	23	0·12	109,296	25	0·11	120,958	33	0·14	118,342	25	0·12
Outer Harbour	30,973	75	1·21	42,456	46	0·54	64,774	39	0·30	74,562	23	0·17
North West	20,412	36	0·88	23,052	52	1·13	34,208	70	1·02	35,230	29	0·46
West	24,478	23	0·47	26,373	26	0·49	34,899	26	0·37	36,134	22	0·34
North East	14,798	110	3·72	16,507	68	2·05	23,472	64	1·36	23,932	37	0·86
Gozo	22,695	37	0·82	23,837	37	0·78	27,680	36	0·65	25,975	28	0·60
Total	207,139	304	0·73	241,521	254	0·53	305,991	268	0·44	314,175	164	0·29

Table 2. Age at onset of disease and type of contact.

	Family contact		Type of contact		
Age of onset	Known	Not known	Mother	Father	Other relative
<20	9 (32%)	43 (68%)	11 (85%)	10 (66%)	22 (63%)
20–29	19 (68%)	20 (32%)	2 (15%)	5 (33%)	13 (27%)
Total	28 (31%)	63 (69%)	13 (21%)	15 (24%)	35 (56%)

urbanization of this former rural area, with socio-economic and hygienic conditions similar to those in the capital. The decline in the highest endemic rural areas came somewhat later. This corresponds with a later improvement in socio-economic and hygienic conditions.

In the last decades leprosy in Malta has become a disease which is largely restricted to a small number of villages 'at the end of the road', where conditions have not yet reached the same high standards as the capital and surrounding area.

In conclusion, largely due to socio-economic and hygienic improvements, the incidence of leprosy had already been declining long before the introduction of effective chemotherapy. The additional effect of chemotherapy is not measurable. Even without chemotherapy it is likely that—as in Norway and other European countries—the disease would cease to be endemic in Malta in the near future. Therefore, Malta does not appear to be a suitable area for demonstrating the eradication of the disease by chemotherapy.

Acknowledgments

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First assessment of the Malta Leprosy Eradication Project

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After the first publications on the Malta Leprosy Eradication Project by Freerksen *et al.*, reporting excellent results and absence of serious side-effects of the drug combination used, doubt about the validity of the data presented still remained. In 1978, four years after most patients had been released from treatment, I was assigned by WHO, as a temporary advisor to the Government of Malta, to make an independent assessment of the project.

This paper concentrates on three questions: 1 Is the patient material in Malta suitable for evaluating the efficacy of a treatment regimen? 2 Were the side-effects as mild as had been reported? 3 Was there any indication of relapses or forthcoming relapses?

The patient material

Most patients had received, prior to the treatment with rifampicin and Isoprodian, prolonged treatment with sulphones. There was also some doubt about the classification of patients. In many patients the disease could have been burned out and then relapse would be unlikely, irrespective of the treatment.

In 1978, in most patients typical symptoms required for classification had subsided, and in many patients it had become difficult or impossible to classify the patients on the basis of clinical symptoms alone. The clinical examination was therefore supplemented by the examination of a skin biopsy and by a lepromin test. Also the clinical records prior to and during the project period were examined. The results of frequent bacteriological examinations of smears (usually every 2–4 months) were available.

On the basis of all these data the patients were classified. There remained no doubt that patients who are classified in this paper as lepromatous, are indeed lepromatous or near-lepromatous. Altogether the status of 86 lepromatous and 46 nonlepromatous patients could be assessed reliably.

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The majority of the patients had received, prior to MDT, prolonged treatment with sulphones and many had been treated with thiacetasone or thiabutosine (Table 1).

However, when MDT was started, a very high proportion of the patients were still bacteriologically positive in smears and/or biopsies. It is of interest that the percentage of bacteriologically positive patients is inversely correlated with the duration of previous treatment. This may be due to decreasing compliance with drug intake. The records showed substantial lack of compliance with drug collection prior to MDT.

Many patients have received low doses of sulphones, often fortnightly sulphone injections. It is therefore probable that many patients harboured sulphone resistant strains of *M. leprae.*

About 50% of the patients classified as nonlepromatous had shown, prior to the onset of MDT, bacteriologically positive smears and 30% were still bacteriologically positive at the onset of MDT. This means that a high proportion of the patients who were classified as nonlepromatous were borderline tuberculoid or borderline, and may have been borderline lepromatous. Only a small proportion were polar tuberculoid.

Of the 86 lepromatous patients, 68 were bacteriologically positive at the onset of MDT. After 24 years of MDT, 66 were still positive. Four years after withdrawal of treatment 42 were found to be bacteriologically positive. In 12 other patients who were still positive the BI had decreased measurably after cessation of treatment (Table 2).

Side-effects

All records were examined. The attendance for drug collection was very regular. During the project period the records were carefully kept. Minor complaints were also recorded. The incidence of side-effects was relatively high. About 25% of the

Table 1. Treatment—and bacteriological status of lepromatous patients at onset of MDT.

No. years treated	No. patients	BI positive at onset of MDT
0–4	17	17 (100%)
5–9	13	7 (54%)
10–19	28	17 (61%)
20+	20	15 (75%)

Table 2. Bacteriological status of nonlepromatous patients.

	BI positive
Time of diagnosis	23 (50%)
Time of onset of MDT	14 (30%)
Time of termination of MDT	0 (0%)

patients had reported gastrointestinal complaints and 13% other complaints such as dizziness (Table 3).

However, the treatment was not withdrawn for any of these patients and no patient stopped attendance because of these complaints. No evidence of serious liver toxicity of the drug combination was found.

Between the onset of MDT and the time of assessment 22 patients had died. This seems to be a high death rate. However, the fact that the average age of the patients in Malta was high should be taken into account. Attempts were made to trace the cause of death (Table 4).

This was not possible in 8 patients. However, all these patients had died only after having completed the course of MDT. Of the 14 other patients the cause of death was traced. In 13 patients no reason was found for assuming a connection with the chemotherapy of leprosy. In one patient, who died of renal failure, the possibility of a relationship with the chemotherapy could not be excluded.

Relapses

On clinical examination no evidence of relapse was found. In none of the biopsies was any cellular activity suggesting a relapse, or forthcoming relapse, seen. In no

Table 3. Side-effects of rifampicin–Isoprodian in 196 patients

Gastric discomfort, nausea, vomiting, eprigastric pain			49 (25%)
Diarrhoea (brief period)			3
Dizziness, vertigo, lassitude, headache			27 (13%)
Skinrash			6
Flush, swelling face			1
ENL	1st year only	27	
	2 years	14	
	3 years	7	
	4 years	3	
	Throughout	19	70
Reversal reactions neuritis			27

Table 4. Cause of death.

Classification	Age	Duration of disease (years)	Cause of death	Years af ter termination of treatment
L	52	> 2	Renal failure	0
L	68	2	Myocardial infarct	0
L	72	> 32	Peritonitis	4
L	46	> 21	Carcinoma mouth	0
L	69	> 16	Hepatic failure (cirrhosis?)	2
L	70	> 6	Heart failure	0
L	71	47	Carcinoma stomach	4
L	42	27	Heart failure	0
L	41	> 12	Carcinoma mammae	2
L	76	31	Carcinoma mammae	0
L	69	> 9	Carcinoma lung	0
L	73	> 11	Unknown	5
L	71	> 12	Unknown	4
L	66	> 30	Unknown	2
L	38	> 9	Unknown	2
L	50	33	Unknown	2
L	70	33	Unknown	1
L	52	> 8	Unknown	2
L	84	> 16	Unknown	3
BL	61	> 14	Cardio-renal insufficiency	2
BT	76	25	Carcinoma mammae	4
T	76	34	Myocarditis	3
T	51	> 18	Carcinoma lung	5

patients, who were found bacteriologically positive in biopsies was a positive morphological index found.

Only one patient with active lepromatous leprosy had shown no bacteriological improvement after MDT, but in this patient the treatment had been very brief and the patient was noncompliant. One nonlepromatous patient showed at the time of the assessment reactivation of pre-existing lesions. This reactive phenomenon was diagnosed as a late reversal reaction.

Conclusion

In a series of patients in Malaysia who had become bacteriologically negative after sulphone monotherapy, about 1% relapse per annum was seen during a follow-up period of 10 years and relapses occurred already in the first four years after cessation of chemotherapy (Waters, personal communication).

In Malta a high proportion of the patients were still bacteriologically positive when chemotherapy was withdrawn.

It is concluded that the patient material in Malta is suitable for comparing the results of treatment with rifampicin–Isoprodian with monotherapy with sulphones. The results in Malta, after a follow-up period of 4 years after cessation of treatment are, in view of the absence of relapses, superior.

In Malta no evidence of serious toxicity of the drug combination was found, but less serious side-effects were common.

Acknowledgments

The author wishes to thank Dr E Bonnici and Dr G Depasquale, who are executing the programme in Malta, for their kind and valuable cooperation, Professor Dr E Freerksen for making available all previous bacteriological data and the Ministry of Health of the Government of Malta for permission to publish the findings.

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A report on two follow-up investigations of the Malta-Project

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Introduction

The objective of the first follow-up investigation, carried out in April 1983, was to examine the multibacillary patients who had been given multidrug therapy (MDT) in Malta since 1972. The particular objectives were the following: 1, to look for signs of clinical relapse; 2, to discover if any leprosy bacilli, whether solid-staining or granular, were present in skin smears; and 3, to study the incidence of side-effects.[1] Professor E Freerksen's design of the Malta-Project was to treat by MDT all leprosy patients whose names appeared on the leprosy register at the Ministry of Health, and similarly to treat all new patients diagnosed in subsequent years, with the objective of eradicating the disease from Malta by rapidly rendering the patients non-infectious:

> 'The Malta-Project is not meant to be a trial with the objective to assess anti-leprosy drugs, but an eradication programme which is exclusively based on antimycobacterial chemotherapy.'[2]

For my part, however, it is as an early and important piece of therapeutic reasearch that I view the Project, and my report does not touch on the eradication aspect.

Two hundred and six patients began MDT in June or July 1972, and included paucibacillary and multibacillary cases. The majority had received monotherapy with dapsone for varying periods measured in years. MDT in all cases consisted of four drugs: rifampicin, dapsone, prothionamide, and isoniazid, the last three being incorporated in a tablet named Isoprodian, and details of dosage and side-effects are given in Dr Depasquale's paper in this series (pp 00–00). The shortest course was 5 months (a BL patient who had previously received dapsone for 6 years), and the longest was 89 months (a new and hyperactive LL patient).

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Materials and methods

There were 128 patients listed as multibacillary who were available for examination in April 1983, 75 males and 53 females, their ages ranging from 20 to 82 years, with a mean of 55 years. Drs E Bonnici and G Depasquale assisted by carrying out some of the clinical examinations, but all skin smears were my responsibility, and I took 6 smears from each of the 128 patients, in the following order:

Smear No. 1 from the right earlobe.
Smear No. 2 from the left earlobe.
Smear No. 3 from the right mid-finger (dorsum of 1st phalanx).
Smear No. 4 from the left mid-finger (dorsum of 1st phalanx).
Smear No. 5 from the right upper arm (just above elbow).
Smear No. 6 from the left upper arm (just above elbow).

Results

Findings in six of the 128 patients were excluded because a study of case records showed that they were paucibacillary throughout. This left a total of 122 multibacillary patients, and from this list a further six were excluded as two had not completed MDT, and four because Dr H Huikeshoven, to whom urine samples were sent for dapsone testing by his ELISA method,[3] could not give an unequivocal assurance regarding absence of dapsone in their urine specimens. Urine samples from the two patients who had not completed MDT were found to be strongly positive. All other specimens were negative.

This left 116 multibacillary patients for clinical and bacterial follow-up, and they consisted of 70 males and 46 females, their ages ranging from 22 to 82 years with a mean of 56. At the time of diagnosis 88 had been classified as LL, 22 as BL, and 6 as BB.

CLINICAL FINDINGS IN 116 PATIENTS

No signs of clinical relapse were found.

BACTERIAL FINDINGS IN 116 PATIENTS

Examination of skin smears was carried out by Dr Marian Ridley at the Hospital for Tropical Diseases, London, and 36 were found to have positive smears (31%); 26 had only granular bacilli (Table 1), and 10 had a few solid-staining bacilli ('solids') (Table 2). In the 10 patients with 'solids', one or other finger was positive in eight, and in seven the fingers were the only sites containing them (Table 2). It is

Table 1. Details of 26 multibacillary patients with only granular bacilli in follow-up skin smears in April 1983

Patient		DDS prior to MDT (years)	MDT began	MDT (months)	Months since beginning MDT	Months since ending MDT
1	LL	3	June 1972	56	130	74
2	BL	7	June 1972	33	130	97
3	LL	24	June 1972	30	130	100
4	LL	19	June 1972	29	130	101
5	LL	11	June 1972	23	130	107
6	LL	19	June 1972	23	130	107
7	LL	17	June 1972	22	130	108
8	LL	5	June 1972	22	130	108
9	LL	29	June 1972	21	130	109
10	LL	20	June 1972	21	130	109
11	BL	5	June 1972	20	130	110
12	LL	19	June 1972	20	130	110
13	LL	5	June 1972	20	130	110
14	LL	26	July 1972	80	129	49
15	LL	21	July 1972	26	129	103
16	LL	14	July 1972	26	129	103
17	LL	23	Sept. 1972	12	127	115
18	LL	17	Nov. 1972	17	125	108
19	LL	15	Apr. 1973	71	120	49
20	LL	1	Feb. 1974	48	110	62
21	BL	0	July 1974	16	105	89
22	LL	2	Mar. 1978	26	61	35
23	LL	3	Aug. 1978	21	56	35
24	LL	0	June 1979	11	46	35
25	LL	0	Mar. 1980	32	37	5
26	LL	0	May 1982	8 (interrupted)	11	3

LL = Lepromatous. BL = Borderline-lepromatous.

possible that these 'solids' represent 'persisters' (drug-sensitive, dormant bacilli), and the generous supply of dermal nerves in fingers increases the likelihood that bacilli sheltering within them may be extracted by the tip of the scalpel blade.

OTHER FINDINGS IN 122 PATIENTS

Routine urine tests revealed protein in seven and sugar in eight. In addition, one specimen contained protein and sugar. The finding of glycosuria in nine patients reflects the high incidence of diabetes in Malta, and the majority of these nine patients were known diabetics.

Table 2. Details of 10 multibacillary leprosy patients with solid-staining bacilli ('solids') in follow-up skin smears in April 1983

Patient		MDT (months)	Months since beginning MDT	Months since ending MDT	Findings in skin smears, April 1983
1	LL	74	130	56	A few 'solids' in left arm. The other 5 smears are negative.
2	LL	72	121	49	One 'solid' in right mid-finger. The other 5 smears are negative.
3	LL	42	83	41	A few 'solids' in left mid-finger, and a few granular bacilli in right arm. The other 4 smears are negative.
4	LL	41	130	89	A few 'solids' and granular bacilli in right earlobe and right mid-finger. The other 4 smears are negative.
5	BL	24	130	106	One 'solid' in right mid-finger. The other 5 smears are negative.
6	LL	23	130	107	One 'solid' in right mid-finger. The other 5 smears are negative.
7	BL	21	56	35	A few 'solids' in left mid-finger. The other 5 smears are negative.
8	BL	20	130	110	A few 'solids' in left mid-finger. The other 5 smears are negative.
9	BL	20	94	74	One 'solid' in left mid-finger. The other 5 smears are negative.
10	LL	14	45	31	A few 'solids' in right arm, and a few granular bacilli in all 6 smears.

In patients 1, 4, 5, 6 and 8, MDT replaced dapsone monotherapy.

THE SECOND FOLLOW-UP INVESTIGATION, APRIL 1986

This involved examining the 10 patients who were found to harbour 'solids' in 1983. No signs of clinical relapse were found, and examination of six skin smears, taken in the same order as in 1983, failed to show bacterial relapse (Table 3). One patient had died, one had no bacilli, five had only granular bacilli, and three had a few 'solids'. Urine was positive for protein in Nos 1, 8 and 10, and sugar was found in No. 5 (a known diabetic). The 'spot test' for dapsone was negative in all.

As regards the 26 patients listed in Table 1, Dr Depasquale informs me that

Table 3. Follow-up skin smears on patients who had 'solids' in April 1983

April 1983			April 1986	
Patient	MDT (months)	Findings	Months since ending MDT	Findings
1	74	Few 's' arm (L)	92	No AFB found
2	72	One 's' finger (R)	85	Few 'gr' ear (R) & both fingers
3	42	Few 's' finger (L) & few 'gr' arm (R)	77	Few 'gr' ear (R)
4	41	Few 's' & 'gr' ear (R) & finger (R)	125	Few 'gr' ear (R) & finger (L)
5	24	One 's' finger (R)	142	Few 'gr' finger (L)
6	23	One 's' finger (R)	143	Few 'gr' ear (L) & finger (L)
7	21	Few 's' finger (L)	71	Patient died, 68 yrs
8	20	Few 's' finger (L)	146	Few 'gr' ear (R); few 's' & 'gr' ear (L)
9	20	One 's' finger (L)	110	Few 's' & 'gr' finger (L); few 'gr' arm (L)
10	14	Few 'gr' in all 6; few 's' arm (R)	67	Few 'gr' ears & fingers; few 's' both ears

's' = 'solids'. 'gr' = granular bacilli

not one has developed any 'solids' over the past 3 years, and the majority now have negative smears. The most significant finding, however, is that I found no evidence of clinical or bacterial relapse in the group who had 'solids' in 1983.

Discussion

These findings raise doubts about the practicality of the recommendation of the WHO Study Group[4] that, where possible, MDT should be continued up to bacterial negativity, namely, up to the time when the last granular bacillus has disappeared from follow-up skin smears, for this may necessitate continuing treatment for 10 years or longer. After all, removing dead bacilli from the tissues is not a function of chemotherapy but is a function of macrophages, and macrophages are peculiarly ineffective at this task in LL. It is hoped that these findings will help in providing an answer to the question of how long MDT should be continued in multibacillary leprosy. It would seem unnecessary to continue up to bacterial negativity, and justifiable to stop treatment at a stage when only granular bacilli are still present in skin smears, so long as clinical improvement is

satisfactory. On the question of whether to stop MDT if 'solids' are present in finger smears, it seems justifiable to stop if: 1, their numbers are small; 2, they have been present over the previous 12 months, i.e. they are not a new discovery; and 3, they are not increasing in number. The important proviso is that at least six post-MDT follow-up smears must be taken every 3–6 months for several years to make sure that they are not becoming more numerous.

Acknowledgments

I wish to thank Professor E Freerksen for permission to report on his patients, Drs E Bonnici and G Depasquale for their cooperation and assistance, and Dr Marian Ridley for reporting on skin smears.

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Report of the Joint Leprosy – Tuberculosis Project in Paraguay

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Following a rewarding initial experience in the treatment of leprosy and tuberculosis with the combination of rifampicin and Isoprodian and taking into account the excellent results of the Malta-Project, the Paraguayan Government, through its Ministry of Health, resolved to initiate an eradication programme of both these diseases which constitute important public health problems in the country.

The realization of the programme was made possible by means of an agreement of cooperation existing between the Government of Paraguay and the German Leprosy Relief Association (DAHW) of Würzburg, and in addition counting on the consultantship—on the technical level—of Professor E Freerksen of the Medical and Biological Research Institute, Borstel.

For the execution of the mixed programme, use was made of the existing infrastructures for leprosy and tuberculosis control.

Concerning the organization of the programme, a central directory, encompassing leprosy and tuberculosis control was created. The Director of the Department for Leprosy Control, Ministry of Health, is in charge of the programme coordination. It should also be mentioned that the leprosy programme is a vertical programme and this proved to be the most efficient way of dealing with the majority of problems at all levels.

Meanwhile the tuberculosis programme is of a horizontal nature, being integrated into the General Health Services.

In the public health approach to both of these diseases there are common characteristics:

1 Treatment is predominantly ambulatory, leaving a small number of patients in need of hospitalization.

1.1 For leprosy patients, under the Ministry of Health, there are many health centres all over the country, the Institute of Tropical Medicine in Asunción, and for cases with advanced incapacities, the leprosarium Santa Isabel of Sapucai.

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1.2 Under the Faculty of Medicine; the relevant wards of the University Hospital in the capital.
1.3 Under private institutions; the Mennonite Hospital km. 81 and the ward of the Patronate for leprosy patients, also in Asunción.
2 For tuberculosis patients:
2.1 Under the Ministry of Health; the Sanatorium Juan Max Boettner.
2.2 Under the Faculty of Medicine, Asunción; the Tuberculosis Service of the University Hospital.
3 In these and other centres patients are not subjected to any kind of segregation and, if necessary, will be attended by other specialists like any other patient. This acceptance by official centres of leprosy patients inspires confidence, thus strengthening the voluntary examination of household contacts and most important, the regularity of treatment.
4 Therapy, including laboratory examinations, is free of charge.
5 The same combined drug therapy is used for both diseases, therefore the Leprosy Service is in charge of drug storage and distribution, safeguarding a close supervision on the utilization of medicaments. For the purpose of the day-to-day management of the joint programme, this has been divided into two sections. First, a leprologist is in charge of the leprosy section, and secondly, a tuberculosis specialist takes care of the TB section. Both these specialists have their offices at the Ministry of Health.

The leprosy section of the joint programme

The following steps were taken during the preparatory phase:

The provision of intensive information on the efficiency of the combined therapy with Isoprodian–rifampicin, mainly to the decision-taking levels of the Ministry of Health. Thus we try to assure the programme support by the highest national authority.

Intensive theoretical and practical training of medical officers, laboratory and nursing staff collaborating with the programme.

Organizing a central dermatological (including leprosy) clinic and a laboratory for clinical and bacteriological tests. These serve as a centre for treatment, training and reference for doctors, nurses, biochemists and laboratory technicians.

Coordinating the programme with government health centres in order to establish skin and leprosy clinics, and, if needed, to provide hospitalization for leprosy patients.

Safeguarding the provision of drugs.

1 INITIATION OF THE PROGRAMME

Officially, the programme was initiated in September 1979 when the first clinic was opened in Asunción for the treatment of leprosy patients with the combination Isoprodian–rifampicin. This clinic, which happens to be the best developed one of the entire programme, functions full-time and is situated in the same building as the Central Health Centre of the Government. Here, highly qualified dermatologists are taking care of leprosy and other dermatological patients. A well-equipped laboratory is functioning, also full-time, in an annex to this clinic and carries out the relevant tests for leprosy patients.

2 PROGRAMME DEVELOPMENT

There was a gradual extension of the programme, linked to the existing public health infrastructure. At this time we have three treatment centres in Asunción and 12 centres in the interior of the country. During the present year, the programme is further expanded to additional towns and villages. To this end, medical officers of government health centres are undergoing a specific training which will enable them to diagnose and treat leprosy patients living inside their area of responsibility.

3 THE CHEMOTHERAPEUTIC COMBINATION USED

During the period 1979–1984, the combination Isoprodian–rifampicin was used in the presentation of two separate dragees. In October 1984, a four-drug combination, namely Isoprodian–RMP was introduced, in one single tablet with the following composition: rifampicin, 150·0 mg; isoniazid, 87·5 mg; prothionamide, 87·5 mg; and dapsone (DDS) 25·0 mg.

This new form of presentation offers the following practical advantages: the combination of substances in adequate proportions inside a single tablet facilitates optimum dosage.

The regular ingestion of the medication by the patient is greatly eased by the fact that he only has to take one tablet at a time.

3.1 *The dosage*

This scheme, which depends on the body-weight, is shown below:

Patients up to	15 kg	take	1 tablet daily
Patients up to	30 kg	take	2 tablets daily
Patients up to	45 kg	take	3 tablets daily
Patients up to	60 kg	take	4 tablets daily
Patients up to	75 kg and above	take	5 tablets daily.

Drug ingestion is daily, from Monday to Saturday, with a break on Sunday.

Duration of therapy is variable, depending upon the clinical and bacteriological progress of each patient. Disregarding in this context the clinical forms (I-B-T-L) of leprosy, the minimum period of treatment is 6 months with an average of about 14 months.

3.2 *The supply of Isoprodian–RMP*

The Department of Leprosy provides Isoprodian–RMP directly to each health centre in accordance with the number of patients on treatment. The drug is deposited under the care of the nursing services and is issued on medical order to the patient at the time of his check-up. Each supply covers a period of 30 days when the patient returns for his clinical and bacteriological controls. The daily drug intake is not supervised since the patient takes medication at home.

The good results we are having with this method of administration are demonstrated by the following facts: the high regularity of patients attending at the clinic or health centre; the favourable clinical and bacteriological evolution of the disease; and the low rate of absconding cases.

4 NUMBER, DISTRIBUTION AND FOLLOW-UP OF LEPROSY PATIENTS TAKING PART IN THE PROGRAMME

A total of 6000 cases are estimated to exist in the country. For the development of the programme it was taken into account that the country has 3258 million inhabitants and that 4957 leprosy patients are in the active register; of these, some 31% of the population and 34% of registered cases are concentrated inside an area of 2852 sq km comprising the city of Asunción and the Central Department.

In the first instance, in September 1979, the work started in these two areas from where it was gradually extended to those parts of the country having the highest demographical concentration and a well-developed public health infrastructure.

In time the programme was expanded to other departments. Since the beginning of the programme, the results obtained with the combination Isoprodian–RMP have been most rewarding, particularly regarding the fast cure of patients followed by the reduction of endemic foci.

The specific programme output has been highly satisfactory. This is best evidenced by the following data: total of estimated cases, 6000; total of registered cases, 4957; under treatment with Isoprodian–RMP, 1623 (27%).

Of these 1623 cases, admitted to the programme, the situation is summed up in Table 1.

Of the 794 cases on observation, 343 were released and taken off the register having completed 3–6 years of post-therapy observation.

It should be mentioned that there are several cases that have been under observation for more than 6 years since ceasing treatment without having

Table 1

Case situation	Number	(%)
Under post-treatment observation	794	(49·0)
Receiving treatment	687	(42·4)
Absconders	80	(5·0)
Deaths	27	(1·6)
Interruptions of therapy	35	(2·0)
Total:	1623	(100·0)

relapsed. The exception is one patient for whom therapy had to be re-started. Of the 27 deaths, not one can be attributed to the specific antileprosy treatment.

The 35 cases which interrupted chemotherapy with Isoprodian–RMP, are accounted for either because of a premature change of medication in a few, mostly private clinics, or of other intercurrent diseases requiring an interruption of specific antileprosy therapy.

The tuberculosis section of the programme

1 OBJECTIVES

The high aims of this programme required the creation of a national tuberculosis register. At present, this covers 53% of the country population incorporating the most populous sanitary regions. New areas are being added in accordance with the planned extension of the programme.

The immediate programme objective is a speedy reduction of the morbidity and mortality rates, aiming at the cure of all cases.

Doubtless, this aim, whose realization is subject to availability of funds, implies a relatively fast programme extension in order to achieve a total population coverage.

2 THE PROGRAMME DEVELOPMENT

The above-mentioned programme aims imply a step-by-step extension, starting in 1979 in the area of the capital and its surrounding populations as well as in several indigenous settlements in the vast Paraguayan area of the Chaco (western region).

In 1981 the programme incorporated the Departments of Paraguarí, Cordillera and the Alto Parana. These areas correspond to the I and IX Sanitary Regions. Other Departments will be added in due course.

In 1984 the TB programme reached a population of about 1·6 million, some 50% of the country's inhabitants.

3 ORGANIZATION

The fight against tuberculosis is integrated into the general health services of the country, implying medical attention for the TB patient by the government health centres which provide the necessary human and material resources.

Regarding the indigenous population, the anti-tuberculosis service can count on the collaboration of the National Association for the Help for the Indigenous Population and, in some instances, is also assisted by paramedical field workers (Promotores de salud).

Concerning the anti-TB service provided by health centres, a medical practitioner attends TB patients once weekly or more often if needed. In the smaller health units the anti-tuberculosis service naturally is of a more limited nature; however it still includes investigations regarding respiratory symptoms, sputum collection and examination by the nearest reference laboratory.

The Central Government Laboratory and the Institute of Tropical Diseases, both in Asunción, serve as TB reference centres and provide the relevant training. In the interior of the country this function is taken over by the Regional Health Centres.

The anti-tuberculosis programme now incorporates 38 localities, including 69 centres providing medical assistance, 22 of which are inside indigenous settlements.

4 PERIOD OF TREATMENT

The short-term therapy with Isoprodian–RMP so far has shown good results. For the majority of patients, treatment is ambulatory, controlled but self-administered.

The minimum period of treatment is five months but this can be prolonged if required by the clinical–bacteriological evolution. This drug-combination is indicated for all forms of the disease, regardless of the extension or severity of the lesion, age, sex or pretreatment. The dosage is the same as that for leprosy patients given and discussed in Section 3.1.

5 COLLABORATION WITH OTHER INSTITUTIONS

The following associations and institutions collaborate together with the Ministry of Health in the national tuberculosis programme: The Paraguayan Institute for the Indigenous population (INDI); The Paraguayan Association for the Indigenous population (API); The Mennonite Association or Cooperative Service to the Indigenous populations (ASCIM); The Anglican Church; The

German Association for Technical Cooperation (GTZ); The Oblatos de Maria; and The Chair for Respiratory Diseases of the Faculty of Medicine, University of Asunción.

6 RESULTS

During the period 1979–1984, 5853 tuberculosis cases were detected, or in other words, 25% of the total estimated number of patients (22,812) existing in the country.

This situation is summed up in Table 2.

The number of patients that abandoned treatment and those who left the area (16·4 and 14·0% respectively), could be explained by the fact that there is a high percentage of migrant people.

Also it could be explained because the information system and the system of registration were not so efficient at the beginning of the programme. The majority of deaths are reported from specialized institutions and refer to advanced cases.

The introduction of the combination Isoprodian–RMP constitutes a most important advance in the fight against leprosy and tuberculosis. Principally this is because of its rapid action and outstanding efficiency, coupled with relatively short periods of treatment.

Table 2

Case situation	Number	(%)
Cured	3135	(53·5)
Abandoned treatment	961	(16·4)
Left the area	821	(14·0)
Died	385	(6·7)
Actually on treatment	551	(9·4)
Total	5853	(100·0)

In the light of these promising results, the ambitious goal of planned disease eradication which has no parallels in other programmes, imposes the need for continuing the ongoing programme until its final realization. To fulfil this goal it is imperative that the joint TB–Leprosy Programme can count on the continuation of the invaluable support of the German Leprosy Relief Association (DAHW). This great challenge has found the backing of the Paraguayan Government and its people almost from the onset of the programme.

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Comparison of DDS with two combined chemotherapy regimens for multibacillary leprosy. Results after 3 years of treatment. A prospective randomized multicentre study.

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Three hundred and seven lepromatous and borderline lepromatous patients in five participating centres (Freetown, Karachi, Bombay, Madras, Chetput) were randomized to receive one of the three following drug regimens: A, DDS 100 mg/day; B, DDS 100 mg/day + Rifa 600 mg/day; and C, Rifa 600 mg/day + Isoprodian (PTH 175 mg, INH 175 mg, dapsone 50 mg), 2 tablets/day. An adjustment was made for patients with less than 60 kg or more than 80 kg of body-weight. Criteria for exclusion included active tuberculosis, any other wasting disease, and psychic disorders. Patients who had been on regular or irregular DDS treatment for 3 years or more were excluded. The main characteristics of the study population are shown in Table 1. A complete physical examination, basic laboratory tests (BUN, GOT, GPT, Hb), skin smears and histology were done before treatment and at regular intervals during the 3 years of treatment. Prior to chemotherapy, a DDS-resistance test was performed and in case of DDS resistance the patient was treated as in Group C, but excluded from the study and evaluated separately. Of the 228 patients who are still in the study 10 (Group D) showed to be fully resistant to DDS.

The study design was to treat patients for 3 years and to have a follow-up period of 5 years. We report the results after 3 years of treatment. Of the 180 cases evaluated so far, a total number of 160 showed regression of leprosy while 20 were clinically classified as stable leprosy. There is no progression. The results for the different treatment groups are shown in Figure 1. The bacteriological index (BI) decreases by the same amount in all treatment groups (Figure 2). Histological serial examinations (number of AFB, staining properties, histopathological status) reveal good therapeutic effect with no statistically different response within groups. A leprosy reaction (Type I or II) was seen in 49%, ranging from 48% in Group A to 64% in Group D. The three drug regimens were tolerated

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Table 1. Characteristics of the three treatment groups

Characteristics	DDS	DDS + RI	ISO + RI
Number at admission	97	108	101
Male/Female	79/16	85/23	82/19
Age (years)			
Mean	32·9	27·9	30·4
BL/LL	40/57	44/64	41/60
BI (mean)	3·6	3·5	3·8
Drop-outs	33	25	20
Remaining for analysis	64	83	81

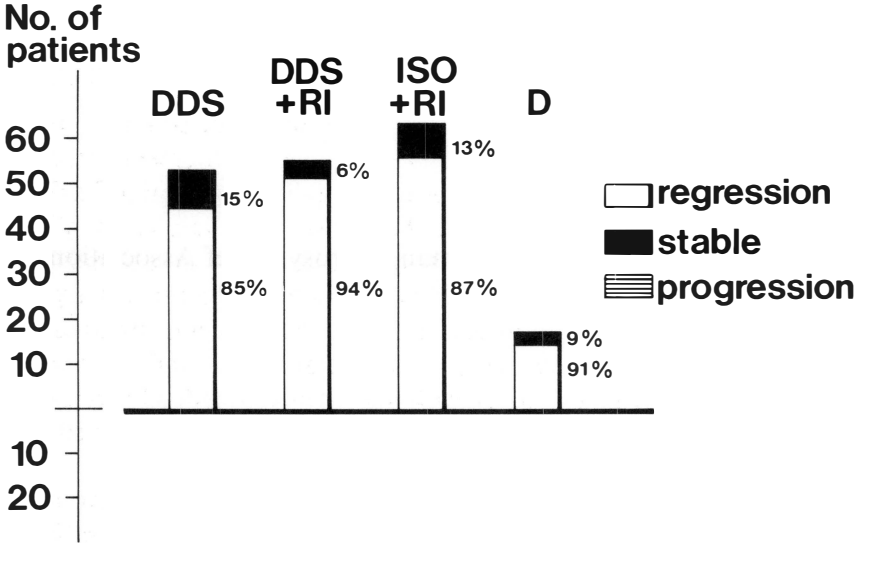

Figure 1. Conclusion of clinical examination after 36 months of treatment

well, and there was no difference in side-effects as judged by GOT, GPT, BUN and haematology serial examinations.

This preliminary evaluation shows no difference in therapeutic response between multidrug and single-drug therapy. Evidence of relapse and/or the development of DDS resistance in the follow-up period will be the important criteria for further recommendations.

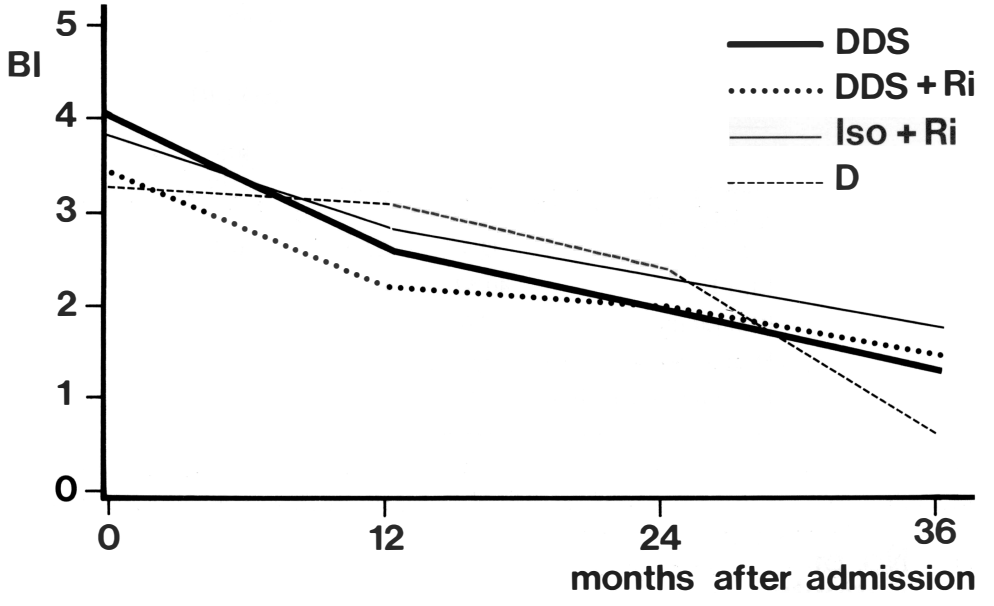

Figure 2. Evolution of the median BI

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The impact of MDT implementation in the Tanzania National TB–Leprosy Programme

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Aware of the world-wide concern about the dapsone monotherapy problem, the National TB–Leprosy Programme (NTLP) has gradually introduced the multiple drug (MDT) treatment of leprosy in its activities. The programme as most of the delegates will recall started in July 1977, and 3 years later MDT was started in some patients thought to be resistant to dapsone.

Since then, the number of patients put on MDT has been gradually increasing from year to year (see Table 1). The new regimen has brought new hope to many old leprosy patients; such that the OPD attendance rate has somewhat increased.

National TB–Leprosy Programme (NTLP)

Just to remind ourselves, NTLP was started in Tanzania to control tuberculosis and leprosy together within the national health services, using the same manpower and transport facilities. The programme was thus entrusted to establish uniform case-finding, case-holding and uniform treatment methods which should be applicable and acceptable for the whole country.

GEOGRAPHICALLY

Tanzania mainland has an area of 930,000 sq km. Situated in the eastern part of Africa, it borders Kenya on the north and Zambia, Mozambique and Malaŵi on the south. It has a population of 21 million, 93% of which is rural.

ADMINISTRATIVELY

The country is divided into 20 regions. Each region is further subdivided into 3–7 districts. There are now 103 districts in the 20 regions. (A region will have 700,000 to 2·5 million population.)

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Table 1. Cases put on MDT 1981–1985

Year	Regions participating	Total no. of cases
1981	3	270
1982	5	554
1983	13	1,566
1984	13	3,027
1985	17	over 4,000

NTLP COORDINATING LEVELS

With such a vast area to supervise and implement MDT, the programme is co-ordinated at 3 levels: (a) the Central level, at the Ministry of Health by two medical officers; (b) the Regional level, by RTLC; and (c) the District level, by DTLC. All of them work together to ensure effective supervision, availability of drugs and other facilities such as transport.

MDT IN 1982 AND ONWARDS

Prior to 1982, cases which were on MDT were put on the WHO regimen of dapsone, clofazimine and intermittent rifampicin. In July 1982, a pilot study was conducted in Dar-es-Salaam to look into the side-effects of Isoprodian and rifampicin combination. Over 300 patients were monitored for about 6 months on an outpatient basis using daily Isoprodian and supervised rifampicin. The patient compliance was good, the attendance rate improved, but the mild side-effects (nausea, dizziness) were somewhat increased. Only one case of mild jaundice was observed.

Table 2. Summary of side-effects noted in various regimen

	No. of patients			Observed side-effects	
Regimen	M	F	Total	Total	%age per group
6 Iso/R	26	10	36	5	13·9
1 R/23 Iso	93	14	107	14	31·1
3 R/21 Iso	25	7	32	13	40·6
24 Iso/R	29	3	32	6	18·8
Total	173	34	207	38	18·4

We thus thought Isoprodian should be tried in Tanzania. Since then, the drug has been used in various combinations to treat both multibacillary and paucibacillary cases.

THE REGIMEN AND SIDE-EFFECTS

(a) For paucibacillary, 6 Iso (R) is mainly used. (b) For multibacillary, admission is generally advised for 1 or 3 months.

Thus: (I) 3 Iso R/ 21 Iso (R); (II) 1 Iso R/ 23 Iso (R) and sometimes (III) 24 Iso (R).

SIDE-EFFECTS

Special efforts to look into side-effects were made. Regional TB/Leprosy Coordinates (RTLC) were requested to fill in special forms for all admitted cases noted to have any side-effects. Nausea and abdominal discomfort were noted in a number of patients. Most of the symptoms disappear after a few days, especially if the drugs were taken after meals. A few patients did not tolerate the drugs because of nausea, vomiting or dizziness. The drugs had to be discontinued. However, jaundice should always be looked for during Isoprodian–rifampicin treatment.

DISCUSSION AND CONCLUSIONS

The introduction of MDT to the NTLP has brought new hope. Many patients who have been on dapsone for years without any hope of recovery now come forward for treatment. The attendance rate has therefore improved.

Urine tests to check for the compliance of self-administration of Isoprodian (for the presence of INH) have been introduced in some regions. According to the evidence we have, so far, the compliance is not 100%. It seems that some patients collect their drugs regularly but do not swallow them daily. This is a big drawback and something has to be done about it soon. The nausea and GTT disturbances might have contributed to this.

There have been a few cases of jaundice, one of them fatal. ENL and reversal reactions have been observed in more patients than on the dapsone monotherapy treatment. They however responded well with analgesics and with a low dosage steroid course.

All in all, the MDT in Tanzania has given more hope to both the patients and the programme administrators. Within 5 months many of the tuberculoid lesions fade away and some of them disappear.

The BI of most multibacillary patients falls to between 1·5 and 2. Many patients do not go beyond 2+ improvement over two years of treatment. Under the light microscope the MIs are invariably zero, the patient clinical improvement

may be evident, but the nodules are still there. However we still stop treatment at this stage (after completion of 2 years treatment within 24–36 months). We are now waiting to see what will happen in these discharged patients. The follow-up continues.

Certainly with the introduction of MDT, intensive patient assessment was carried out, i.e. clinical and bacteriological examination of all patients. This exercise has had direct impact on the reduction of the number of registered cases.

In 1979, for example we had 50,740 registered cases. With the introduction of NTLP and implementation of MDT, the caseload is now 30,200.

The future of leprosy in Tanzania will probably be increasingly concerned with the care of crippled patients (and a few new patients), trying to rehabilitate them and make them acceptable to society. It is important to remember that a deformed and disabled patient does not consider himself to be 'cured' and that society views such a patient as a true case of leprosy.

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Effect of clofazimine and dapsone on rifampicin (Lositril) pharmacokinetics in multibacillary and paucibacillary leprosy cases

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Introduction

WHO Expert Committee on Leprosy has recommended combined chemotherapy for both multibacillary and paucibacillary leprosy cases with an objective of preventing the multiple drug resistance consequent to emergence of wide-spread dapsone resistance (both primary and secondary). It has also been demonstrated that daily administration of 600 mg rifampicin is not more effective than monthly administration of 600 mg on consecutive days.[8]

The peak blood levels of rifampicin in normal adults vary widely from individual to individual and also within the same subject. Peak blood levels occur between 2 and 4 hr following the oral administration of 600 mg dose. The average peak level is 7 mcg per ml; however, the peak level may vary from 4 to 32 mcg per ml. In normal adults the $t\frac{1}{2}$ of rifampicin in blood is approximately 3 hr. Elimination occurs mainly through the bile and to a lesser extent by the urine.[1]

Abbreviations used in the article: MCR, metabolic clearance rate (ml/mt/kg = millilitre per minute per kilogram body weight); Ke, rate constant for elimination (h^1 = rate per hour); Auc, area under curve (μg-h/ml = microgram hours per millilitre); Cmax, maximum concentration (μg/ml = microgram per millilitre); RC, rifampicin + clofazimine; RDC, rifampicin + dapsone + clofazimine; RD, rifampicin + dapsone; Ka, rate constant for absorption (h^1 = rate per hour); avd, apparent volume of distribution (l = litre); avd-kg, apparent volume of distribution per kilogram body weight; avd-kg(l), apparent volume of distribution per kilogram body weight expressed in litres; tp, time to reach peak concentration; $t\frac{1}{2}$, half life; and Auc/$t\frac{1}{2}$, ratio of area under curve to half life.

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To what extent dapsone and clofazimine can affect rifampicin pharmacokinetics has not been studied in cases of multibacillary and paucibacillary leprosy.

Objectives of the present study

With the following three objectives, the present study was initiated:

1 To determine to what extent the type of leprosy (multibacillary and paucibacillary) can influence pharmacokinetic parameters of rifampicin.
2 To study the influence of following three regimens on the pharmacokinetic parameters of rifampicin (within comparison): (a) rifampicin + dapsone + clofazimine (RDC); (b) rifampicin + dapsone (RD); and (c) rifampicin + clofazimine (RC).
3 To determine differences in pharmacokinetic parameters of rifampicin within the above three groups, i.e. in between comparison; RDC *vs* RD; RDC *vs* RC; and RD *vs* RC).

Materials and methods

SUBJECTS

Male leprosy cases in the age group of 15–60 years were selected. Selection criteria used are enumerated below:

1 *Inclusion.* a, non-obese patients; b, a confirmed case of multibacillary or paucibacillary leprosy; and c, should not have received any other drugs for at least 2 weeks prior to initiation of the study.

2 *Exclusion.* a, History of allergy to any one of the drugs in study period, i.e. rifampicin, dapsone and clofazimine; b, patients having any gastrointestinal ulcers and history of gastrectomy; c, patients requiring any other concomitant therapy; and d, patients with any other disease, haematological, renal, cardiovascular or hepatic.

Plan and design

METHOD

Complete double blind within comparison design was used. A minimum time span of 2 weeks between doses, after an initial 'run in' period of one week, was

Table 1. Experimental design.

Pre-regimen phase			Post-regimen phase		
Week	Day		Week	Day	
I	Wednesday	$RDC_1:RD_1:RC_1$	III	Wednesday	$RDC_1:RD_1:RC_1$
	Friday	$RDC_2:RD_2:RC_2$		Friday	$RDC_2:RD_2:RC_2$
II	Monday	$RDC_3:RD_3:RC_3$	IV	Monday	$RDC_3:RD_3:RC_3$
	Wednesday	$RDC_4:RD_4:RC_4$		Wednesday	$RDC_4:RD_4:RC_4$
	Friday	$RDC_5:RD_5:RC_5$		Friday	$RDC_5:RD_5:RC_5$
III	Monday	$RDC_6:RD_6:RC_6$	V	Monday	$RDC_6:RD_6:RC_6$

kept. The allocation of drug regimen was based on nature of leprosy. Specimens were code labelled as per master code sheet. Details of experimental design are given in Table 1.

DOSAGE

'Run in' period. The patients were divided into three groups of six patients each. Physical examination (height, weight and midarm muscle circumference) and laboratory investigations such as bacteriological index, nasal smear, blood (total and differential WBC counts, haemoglobin, haematocrit, bleeding time, coagulation time and platelet count), urine, stool and sputum examination, liver function tests (SGOT, SGPT, alkaline phosphatase and serum bilirubin), were carried out and recorded in the proforma. No drugs were given during this period of one week.

Pre-dose period. Patients were fasted overnight for 8 hr prior to the day of estimating the drug levels. One hour prior to the study, 250 ml of water was given to each of them. They were asked to void urine 15 min prior to the dose administration.

Dosing. Both multibacillary and paucibacillary cases received 600 mg rifampicin alone at the exact time of drug administration. The time at which rifampicin had been given was recorded for each of the patients.

Post-dosing period. The cases were not given any food during the next 3 hr of the study, but 150 ml of water was given at the end of 1 and 2 hr. No strenuous or athletic activity was permissible during the period of study. A light breakfast was given 3 hr after the study, i.e. at 12.15 pm.

PHARMACOKINETIC SAMPLING

A siliconized intravenous catheter was used for withdrawing 3 ml of blood in a 15 ml code labelled centrifuge tube at 9.00 am, 10.00 am, 11.00 am, 1.00 pm and 3.00

pm on the first day and 9.00 am on the next day; i.e. 0, 1, 2, 4, 6 and 24 hr blood samples were collected. Any deviations from the stated sampling times were recorded on the form. For withdrawing blood, Medivik disposable sterile syringes were used. The code labels and code letters were selected by the investigator for each case.

The separated serum was transferred to code labelled vials. The vials were sealed with aluminium seals by a hand-sealing machine. Until the time of transportation, they were kept in a Thermos with ice in the refrigerator. On the day of transportation, they were taken in the Thermos flask. On the same day the microbiological assay of rifampicin was done by the USP method.

STATISTICAL ANALYSIS

Using a single compartment pharmacokinetic model the values of Ka, Ke, Auc, avd^{-kg}, MRC, $t\frac{1}{2}$ tp., Cmax and Auc/$t\frac{1}{2}$ were calculated. Mean and standard error for the pharmacokinetic parameters were calculated. Student's 't' test (two tailed) was used for 'in between comparison' and paired 't' test was used for 'within comparison'.

Results

COMPARISON OF PHARMACOKINETIC PARAMETERS IN MULTIBACILLARY AND PAUCIBACILLARY LEPROSY CASES

No statistically significant difference was noted in rifampicin pharmacokinetic parameters, like Ka, Ke, Auc, avd^{-kg}, tp, MCR, $t\frac{1}{2}$, Cmax and Auc/$t\frac{1}{2}$ in multibacillary and paucibacillary leprosy cases. Apparently the type of leprosy has no effect on rifampicin pharmacokinetics.

EFFECT OF DIFFERENT REGIMENS ON THE RIFAMPICIN PHARMACOKINETICS (WITHIN COMPARISON)

The following observations were noted:

1 *RC Group*. While comparing 'within patient' data for the pre- and post-regimen phases of RC group, clofazimine was found to reduce the rate of absorption (Ka) of rifampicin ($P<0{\cdot}01$) and delay the time to reach the peak serum concentration (tp) ($P<0{\cdot}01$). However, no statistically significant difference was noted in other pharmacokinetic parameters, i.e. Ke, Auc, avd^{-kg}, MCR, $t\frac{1}{2}$, Cmax and Auc/$t\frac{1}{2}$ ratio.

2 *RDC Group*. Except for reducing Auc ($P<0{\cdot}05$), no statistically significant difference in other pharmacokinetic parameters of rifampicin, i.e. Ka, Ke, avd^{-kg}, MCR, $t\frac{1}{2}$, tp, Cmax and Auc/$t\frac{1}{2}$ ratio was noted in group RDC. However, 1 h

Table 2. Effect of clofazimine, dapsone and combinations (clofazimine plus dapsone) on serum concentration of rifampicin (Lositril).

Group		1h	2h	4h	6h
RDC	Pre	6·15±1·32 ($n=6$)	5·52±6·97 ($n=6$)	4·9±0·55 ($n=6$)	3·81±0·45 ($n=6$)
	Post	2·66±1·35* ($n=6$)	7·02±0·86 ($n=6$)	5·43±0·78 ($n=6$)	3·34±0·60 ($n=6$)
RD	Pre	6·4±0·75 ($n=6$)	5·83±0·55 ($n=6$)	4·25±0·63 ($n=6$)	3·05±0·38 ($n=6$)
	Post	3·7±1·86 ($n=5$)	7·78±1·34 ($n=5$)	4·78±0·74 ($n=5$)	3·22±0·53 ($n=5$)
RC	Pre	3·95±0·98 ($n=6$)	5·85±0·57 ($n=6$)	5·51±0·42 ($n=6$)	3·40±0·80 ($n=6$)
	Post	3·82±0·84 ($n=5$)	5·6±1·38 ($n=5$)	5·85±0·72 ($n=4$)	4·26±0·72 ($n=5$)

NB, n, number of patients; RDC, rifampicin + dapsone + clofazimine; RD, rifampicin + dapsone; RC, rifampicin + clofazimine; values are expressed as mean ± SE.

* $P<0·05$.

Paired 't' test used for 'within comparison'

serum concentration of rifampicin was significantly reduced in RDC group ($P<0·05$) in post-regimen phase.

3 *RD Group*. No statistically significant alteration in any of the pharmacokinetic parameters of rifampicin was noted in group RD.

COMPARISON OF PHARMACOKINETIC PARAMETERS OF RIFAMPICIN IN PRE- AND POST-THERAPY PHASES IN VARIOUS GROUPS (IN BETWEEN COMPARISON)

1 While comparing the three groups ('in between patient' data), no statistically significant difference was noted when RDC was compared with RD. In the post-regimen phase of RC, the following statistically significant differences in pharmacokinetic parameters of rifampicin were seen as compared to RDC:

(a) The rate of elimination of rifampicin in RC was significantly less than in the RDC ($P<0·05$).

(b) The metabolic clearance rate of rifampicin in RC was significantly less than in the RDC ($P<0·02$)

(c) The $t\frac{1}{2}$ of rifampicin in RC was significantly more than in the RDC ($P<0·02$)

(d) The Auc/$t\frac{1}{2}$ ratio of rifampicin was significantly more in the pre-regimen phase

Table 3. Effect of clofazimine, dapsone and combination (clofazimine & dapsone) on pharmacokinetic parameters of rifampicin (Lositril).

Therapeutic regimen	n	Ka (h^{-1})		Ke (h^{-1})		Auc (μg-h/ml)		avd (l)	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Group RDC	6	0·650 ±0·082	0·436 ±0·011	0·159 ±0·013	0·209 ±0·021	48·55 ±5·29	*35·43 ±7·45	87·74 ±17·21	96·19 ±13·30
RD	5	0·675 ±0·066	0·522 ±0·049	0·159 ±0·023	0·199 ±0·025	43·84 ±6·08	44·02 ±8·09	93·74 ±4·13	81·74 ±15·52
RC	5	0·566 ±0·055	†0·389 ±0·024	0·191 ±0·023	0·165 ±0·009	51·05 ±2·48	55·23 ±7·19	64·69 ±6·06	78·88 ±10·56

Therapeutic regimen	n	avd-kg(l)		MCR (ml/mt/kg)		$t\frac{1}{2}$		tp (h)	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Group RDC	n	1·86 ±0·38	2·06 ±0·79	0·0046 ±0·0006	0·0059 ±0·0009	4·51 ±0·41	3·44 ±0·30	3·09 ±0·40	3·46 ±0·38
RD	5	1·79 ±0·10	1·59 ±0·33	0·0048 ±0·0009	0·0049 ±0·0009	4·66 ±0·55	3·64 ±0·35	2·92 ±0·27	3·09 ±0·27
RC	5	1·27 ±0·16	1·47 ±0·11	0·0037 ±0·0	0·0040 ±0·0002	3·80 ±0·36	4·23 ±0·25	2·95 ±0·16	3·84 ±0·12

Therapeutic regimen	n	Cmax (μg/ml)		Auc/$t\frac{1}{2}$	
		Pre	Post	Pre	Post
Group RDC	6	6·78 ±0·84	5·28 ±0·73	11·20 ±1·43	10 ±1·49
RD	5	6·15 ±0·26	8·01 ±2·3	9·30 ±0·41	12·85 ±3·02
RC	5	7·32 ±0·25	6·23 ±0·66	13·94 ±1·51	12·15 ±2·24

Values expressed as mean ± SE; R, rifampicin; d, dapsone; c, clofazimine.

* Denotes $P < 0·05$

† Denotes $P < 0·01$

Paired 't' test used for 'within comparison'.

of RC than in RDC ($P < 0{\cdot}05$); while mean Auc/$t\frac{1}{2}$ ratio was less in 'post-regimen phase' of RC than 'pre-regimen phase' value and the 'post regimen' mean Auc/$t\frac{1}{2}$ ratio was more in RD than the 'pre-regimen phase' value; but no statistically significant difference was noted. While dapsone showed the tendency to increase Auc/$t\frac{1}{2}$ ratio, clofazimine reversed this tendency and the overall effect in the RDC group was the tendency to reduce Auc/$t\frac{1}{2}$ ratio.

2 While comparing pre-regimen phases of RD with RC, the following statistically significant differences were noted:

(a) The rate of absorption of rifampicin was significantly less in RC than in RD ($P < 0{\cdot}05$).
(b) The apparent volume of distribution of rifampicin was significantly more in RD than in RC ($P < 0{\cdot}001$). The avd$^{(l/kg)}$ was also significantly different in the same way ($P < 0{\cdot}01$).

3 Similar comparison of post-regimen phase of RD with RC, following changes in the pharmacokinetic parameters of rifampicin were noted:

(a) The rate of absorption of rifampicin was further significantly reduced in RC than in RD. The degree of statistical significance was also increased ($P < 0{\cdot}05$ to $P < 0{\cdot}01$).
(b) While the avd in RD regimen reduced from $93{\cdot}74 \pm 4{\cdot}13$ to $81{\cdot}74 \pm 15{\cdot}52$, it increased in RC regimen from $64{\cdot}69 \pm 6{\cdot}06$ to $78{\cdot}88 \pm 10{\cdot}56$. Hence no statistically significant difference was noted.
(c) The time to reach peak serum concentration (tp) was significantly more in RC than in RD ($P < 0{\cdot}01$). The $t\frac{1}{2}$ was also increased though not significantly statistically.
(d) While Cmax increased in RD regimen from $6{\cdot}15 \pm 0{\cdot}26$ to $8{\cdot}01 \pm 2{\cdot}3$ it declined in RC from $7{\cdot}32 \pm 0{\cdot}25$ to $6{\cdot}23 \pm 0{\cdot}66$. A similar trend was seen in Auc/$t\frac{1}{2}$ ratio. Hence no statistically significant difference was noted.

Discussion

In the present study, rifampicin pharmacokinetics was not affected by the type of leprosy. However, it is evident that the absorption of rifampicin was relatively less than that seen in healthy human volunteers. Whether it is due to reduced gastrointestinal absorption due to the disease *per se* or due to lower levels of plasma proteins is difficult to say due to the paucity of data for these assumptions.

Viratenen & Tala (1974)[7] have reported that prolonged treatment increases the rate of inactivation or elimination of rifampicin. Since rifampicin has been given only as a single dose, the possibility of it *per se* increasing the rate of its inactivation or elimination is ruled out.

Dapsone did not affect the rifampicin bioavailability, and this finding is

consistent with experimental observations in mice where no evidence of any antagonism between dapsone and rifampicin has been demonstrated.[5] Rifampicin is capable of increasing the rate of clearance of dapsone from plasma, possibly secondary to the induction of hepatic microsomal enzymes that may increase N-hydroxylation (which is microsome dependent) instead of increasing the acetylation of dapsone.[2,3] Furthermore, the levels of dapsone achieved in blood by means of daily doses of 50–100 mg are far too high to be reduced to subtherapeutic levels by sustained daily treatment with rifampicin. Clearly then, the employment of a once monthly schedule of rifampicin administration exerts little if any effect on the rate of dapsone clearance. In the present study the levels of dapsone were not estimated, but it can be safely concluded that regular dapsone administration did not alter the rifampicin pharmacokinetic parameters. Thus concomitant combined regimen of dapsone with rifampicin does not pose a pharmacokinetic drug interaction problem in the management of leprosy patients.

To what extent single administration of rifampicin and daily administration of dapsone can influence clofazimine levels in leprosy cases has not been studied in the present study, but effect of dapsone and clofazimine alone and in combination has been assessd on the rifampicin pharmacokinetic parameters.

Except for significant reduction in Ka ($P<0{\cdot}01$) and an increase in tp ($P<0{\cdot}05$) in RC group and significant reduction in 1-hr serum rifampicin level ($P<0{\cdot}05$) and Auc ($P<0{\cdot}05$) in RDC group, no other statistically significant alterations were produced perhaps due to opposing trends in other parameters.

Although not statistically significant, two basic opposing trends were observed. While clofazimine showed the tendency of increasing the apparent volume of distribution coupled with a tendency to reduce the rate of elimination, dapsone exerted an opposing influence. Since clofazimine reduced Ka significantly in addition to the trend of increasing avd and MCR, the logical outcome should have been a reduction in $t\frac{1}{2}$ and Auc, but on the contrary a trend of increase was noted, perhaps best explained by the trend of reducing the rate of elimination.

On the other hand, dapsone reversed the trends in the opposite direction. A trend of reduced apparent volume of distribution associated with a tendency to increase the rate of elimination was seen. Thus the former resulted in a trend of increase in Cmax and Auc of rifampicin, while the latter, in addition to an increase in MCR, accounted for a trend of increase in $t\frac{1}{2}$ of rifampicin; thus overall effect was negligible.

Consequent to these opposing influences, combined effect of dapsone and clofazimine resulted in a trend of reduction in Ka and increase in tp of rifampicin. Added to this was the trend of an increase in MCR, avd and Ke accounting for the tendency of decrease in Cmax and $t\frac{1}{2}$ and overall statistically significant reduction in Auc ($P<0{\cdot}05$).

Of the three groups, both RDC and RD were homogenous groups. Auc/$t\frac{1}{2}$

ratio of rifampicin in RC group was comparatively higher than in RDC ($P < 0{\cdot}05$) and RD group ($P < 0{\cdot}001$). While comparing with RD group rifampicin avd and Ka were relatively less ($P < 0{\cdot}001$; $P < 0{\cdot}05$ respectively) in RC group, the former resulting in increased Cmax ($P < 0{\cdot}01$) despite a reduced Ka and thus resulting in an increased Auc/$t\frac{1}{2}$ ratio even in the pre-regimen phase ($P < 0{\cdot}01$).

On the other hand except for Auc/$t\frac{1}{2}$ ratio ($P < 0{\cdot}05$), both RDC and RC groups were homogenous in the pre-regimen phase. And in post-regimen phase while clofazimine showed the tendency of reducing rifampicin Ka ($P < 0{\cdot}05$) and increasing MCR ($P < 0{\cdot}02$), dapsone was found to increase both the parameters. And this effect predominated even when dapsone was used concomitantly with clofazimine; thus RDC differed significantly from RC in these respects. Ultimately, it resulted in a prolonged $t\frac{1}{2}$ in RC group significantly different from RDC group ($P < 0{\cdot}02$).

Conclusions

Clofazimine reduced rifampicin absorption significantly resulting in delayed time to reach peak serum concentration and increased $t\frac{1}{2}$, but no significant change was seen in Auc, and Cmax because Ke was reduced significantly by clofazimine when used without dapsone in addition to insignificant increase in MCR.

Obviously, further studies are required to determine the time interval to be kept between administration of clofazimine and rifampicin so that it may not interfere with the absorption of rifampicin. In view of this, the following questions were raised:

(a) Whether it is appropriate to give clofazimine daily when it is known to have a long half life.
(b) Whether it would be rational to combine the monthly supervised dose of clofazimine (300 mg) with rifampicin (600 mg) in the light of the present findings. In view of this, whether it would be appropriate to increase the monthly dose of rifampicin to compensate for the 'clofazimine effect'.

Some clinical impressions of Multidrug therapy (MDT) in leprosy

The Poona District Leprosy Committee is running two leprosy control projects, one in the urban area of Poona and the other at Solapur. In these projects, by the end of December 1985, 4532 leprosy patients were given MDT. Of these, 1828 patients are multibacillary while 2704 are paucibacillary.

The standard regimen recommended by the WHO Technical Report Series 675[9] is being followed for multibacillary as well as paucibacillary cases. The patients who have maintained regularity in treatment have shown clinical as well

as bacteriological improvement. They did not show major side-effects/toxicity. A detailed analysis of the data is being undertaken. However, a few cases in whom interesting clinical findings were observed are reported here.

In 21 multibacillary cases, the bacteriological index started falling initially and after it dropped to 1·0/1·5 it remained static, even after continuing MDT, though they showed definite clinical improvement. The pigmentary changes and the ichthyotic changes seen during clofazimine therapy persist for more than 2 years after stopping clofazimine. In a few patients, pigmentation due to clofazimine therapy is not pronounced, though supervised clofazimine therapy is confirmed. The reason for this is not known.

In 20 paucibacillary patients, those previously treated with dapsone monotherapy and later put on MDT did not show much clinical improvement in respect of the patches, while clinical response in new untreated paucibacillary patients treated with MDT is excellent, as observed in the disappearance of the patches. It is the observation of some of our colleagues that if MDT is discontinued after 6 months, patients report back within 1 year with relapse in the form of new patches.

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Experience with multidrug therapy in Sierra Leone: clinical, operational and managerial analysis

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Introduction

MDT as recommended by WHO was introduced in March 1983. For the multibacillary cases the combination with clofazimine is used.

In January 1986, 1745 paucibacillary (PB) cases and 688 multibacillary (MB) cases were put on MDT. So far 1192 PB cases have been discharged after completing 6 months, MDT. None of the MB cases have been discharged yet.

Four hundred and forty-six PB cases who should have completed 6 to 33 months of follow up and 105 MB cases who should have completed 12 to 33 months of treatment are analysed here. Of the 464 cases 124 were TT, 325 were BT, 14 were BB and 1 was I.

MDT was discontinued in 95 (20·4%) patients who failed to attend the clinics for 4 or more times. Three hundred and sixty-nine patients (79·6%) completed the full treatment within 6–9 months.

Looking through 15 years of annual reports of the Leprosy Control Programme, we found 28% of the known PB cases were recorded as out of control. In the remaining 72% of PB cases only 60 to 70% completed the full course of treatment (regular) before being discharged as cured.

From Table 5(a) and (b) it is seen that: The patients with single lesions tend to drop out more, compared to any other group. Presence of thickened nerve, deformity and ulcer makes the patients more regular. These two facts are also true with DDS monotherapy.

Table 6 shows that: 90% of the cases became inactive within a 6-month period. Many cases became inactive between 3 and 5 months. However 10% of the cases remained active at the end of a 6-month period. Similar monthly observation during the first 6 to 9 months of DDS monotherapy could not be found.

However, observation on a large number of PB cases at Dharmapuri, India[1] gives the following data: Time taken for PB cases to become inactive: 1, few skin

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Table 1. The status of the disease at commencement of MDT.

	TT	BT	BB	I	Total
Active	33	113	3	—	149
Inactive*	91	212	11	1	315
Total	124	325	14	1	464

* Inactive cases who needed more than 12 months of DDS maintenance therapy were put on MDT.

Table 2. Break up according to number of skin lesions.

	No. of patches					
	Single	5	5–15	15	No skin lesion	Total
Active	38	38	57	16	—	149
Inactive	79	95	98	32	11	315
Total	117	133	155	48	11	464

Table 3. The number of patients who had thickened nerve alone, thickened nerve + deformity and ulcers.

	Thickened nerve alone	Thickened nerve + ulcer	Thickened nerve + deformity	Thickened nerve + deformity + ulcer
Active	14	3	17	3
Inactive	33	4	22	16
Total	47	7	39	19

lesions, 179 weeks; 2, multiple skin lesions, 200 weeks; 3, few and thickened nerve, 181 weeks; and 4, multiple and thickened nerve, 205 weeks.

It appears DDS monotherapy needs a much longer period of treatment to make an active case inactive, compared to MDT.

However in the absence of monthly follow ups during the first 6 to 9 months of treatment with DDS monotherapy, no definite conclusion could be drawn.

Table 4. Attendance.

Treatment	Active	Inactive	Total
Discontinued	35	60	95 (20·4%)
Completed in 6 months	92	177	269 (79·6%)
Completed in 9 months	22	78	100
Total	149	315	464

Table 5(a). Patients discontinued: relationship with number of patches.

No. of skin lesions	Single	5	5 to 15	15	No skin lesion	Total
Active	9	10	14	2	—	36
Inactive	29	8	24	2	2	60
Total	34 (29%)	18 (14%)	38 (25%)	4 (8%)	2 (18%)	(20·4%)

Table 5(b). Patients discontinued; relationship to thickened nerve, deformity and ulcer

	Thickened nerve	Thickened nerve +ulcer	Thickened nerve +deformity	Thickened nerve +deformity +ulcer
Active	3	—	4	1
Inactive	1	—	—	—
Total	4 (7·4%)	—	4 (6·8%)	1

Table 6. Time taken by the active cases to become inactive.

Months	1	2	3	4	5	6	Remained active at 6 months	Total
No. of cases	9	14	18	17	27	17	12	114
	8%	12%	16%	15%	24%	15%	10%	

Table 7(a).

	Single	< 5	5 to 15	> 15	Total
Became active at 6 months	27	23	23	13	102
Remained active at 6 months	2	5	4	1	12
Total	29	28	43	14	114

Table 7(b).

	Thickened nerve alone	Thickened nerve + deformity
Became inactive within 6 months	14	18
Remained active at 6 months	3	2

On further analysis it is found that there is no definite relationship between the number of patches and the time needed to make them inactive.

It appears that the involvement of nerve slightly prolongs the time duration; 13·5% of active cases with nerve involvement remained active at the end of 6 months treatment compared to 9·1% of active cases without nerve involvement which remained active at the end of 6 months treatment.

Follow up

Altogether 14 out of 237 cases who came for follow up relapsed (relapse rate 5·9%). The relapse rate among active cases is higher, 12·3% compared to inactive cases 3·04%. Out of 369 patients 132 never attend follow up clinics (dropout rate of 35%). Varying relapse rates among PB cases have been reported with DDS monotherapy from 1·8%[1] to 15%.[2] All the relapses have occurred within the first 18 months of follow up.

Eight out of 14 relapsed cases had 5 to 15 patches to start with. No definite relationship between the number of patches and number of relapse.

Active cases with nerve involvement relapsed more than the active cases without nerve involvement. The trend is opposite in inactive cases.

Percentage wise there is more relapse in BB followed by BT and very little in TT. There is no relationship between treatment irregularity and relapse, since all the relapsed cases completed their treatment in 6 continuous months.

No relapse has occurred in those patients who had typical atrophic, wrinkled, scarred lesions at the end of treatment. All the relapses have occurred in those who had residual, hypopigmented, vague lesions, but not actual scar and in those the skin lesions have totally disappeared.

Table 8.

Follow up in months	Inactive	Relapse	Active	Relapse
Never came for follow up	91	?	41	?
3	36	3	18	5
6	25	2	9	2
12	34	Nil	9	1
18	34	—	13	1
24	30	—	10	
30 and above	5	—	14	
Total	255	5	114	9

Table 9(a).

	Single	<5	5 to 15	>15	Total
Active	—	1 (3·5%)	6 (18·1%)	2 (14·2%)	9
Inactive	3 (5·5%)	—	2 (2·7%)	—	5
Total	3 (3·6%)	1 (0·86%)	8 (7·47%)	2 (4·5%)	14

Table 9(b).

	With nerve involvement	Without nerve involvement	Total
Active	4 (13·3%)	5 (5·9%)	9
Inactive	1 (1·35%)	4 (2·2%)	5
Total	5 (4·8%)	9 (3·3%)	14

Table 10(c).

	TT	BT	BB	I	Total
Active	—	8 (9·6%)	1 (33·3%)	—	9
Inactive	1 (1·1%)	4 (2·5%)	—	—	5
Total	1 (0·8%)	12 (5%)	1 (7·14%)	—	14

Table 11. The status of skin lesions at the end of treatment and the relapse.

Scarred		Residual		Disappeared		
Total	Relapse	Total	Relapse	Total	Relapse	Remained active
65	Nil	222	11 (4·9)	70	3 (4·2%)	12

The scarred lesions are probably the end result of high CMI reaching hypersensitive proportions. This is supported by the fact that Type I reaction treated with steroids invariably leaves a typical scar.

Whether the useful CMI and hypersensitivity are the same or not, whether one is associated with the other or not; it seems CMI bordering on to hypersensitivity level is needed for complete resolution of lesions and probably complete elimination of antigen.

Six out of the 14 cases that relapsed, relapsed with Type I reaction (42·8%).

Type I Reaction occurred without, with and after stopping treatment, and occurred in both active as well as inactive cases. Further analysis showed that

Table 12. Relapse and Type I reaction.

	Relapsed with	Relapsed with	Total
Active	2	7	9
Inactive	4	1	5
Total	6 (42·8%)	8 (57·2%)	14

Table 13. Type I reaction (eight patients with nine reactions).

	Came with	During treatment	After stopping treatment	Total
Active	1	1	2	4
Inactive	—	1	4	5
Total	1	2	6	9

there is no relationship with the number of initial lesions. Nerve involvement, duration of treatment and regularity of treatment. Type I Reaction has been reported in those who never had treatment.[3] It appears that the Type I reaction is a natural phenomenon in borderline leprosy to the dead bacterial antigen released to a threshold level. Antileprosy drugs caused release of dead antigen, one might expect a large number of Type I reactions with bacterioxidal drug rifampicin as compared to DDS monotherapy and in those who never had any antileprosy drugs. But this seems to be not true. It seems other factors like the amount, the type and the time of release of antigen and the immunological status of the patient at that time plays a major role in the production of Type I reaction.

Table 14(a). Follow up of cases who had Type I reaction.

	Type	Time of onset of reaction	Results after treatment	Follow up
Active	BT	3rd month during MDT	Residual	Relapsed 16 months after with active lesions
	BT	2nd month during MDT 11th month after stopping MDT	Still on steroids	Following the 2nd episode
	BT	Came with Type I	Residual	3 months O.K.
	BT	4th month during MDT	Scarred	Never came for follow up
Inactive	BT	3rd month during MDT	Scarred	20 months O.K.
	BT	2nd month after stopping MDT	Scarred	15 months O.K.
	BT	3rd month after stopping MDT	Scarred	11 months O.K.
	BT	9th month after stopping MDT	Scarred	3 months O.K.

All the Type I reactions were treated with steroids (low to moderate dose) for 3 to 6 months depending on clinical response. All responded well, 5 ending in scarred lesions, 2 with residual lesions. Follow up shows 2 relapsing 11 and 16 months after stopping steroids. One relapsed with Type I reaction and the other with active lesions.

Follow up of relapsed cases

Six out of 14 relapsed cases relapsed with Type I reaction, the follow up of these are given above. The follow up of the remaining 8 cases are given in Table 14(b).

No treatment was given to 5 patients, but they were observed monthly. Two became inactive, 7 and 3 months after relapse and remained inactive at 3 and 8 months follow up. Two are still active at 6 and 1 month after relapse. One patient relapsed with BB, was found to have hepatic malignancy and died 2 months after. Three patients who remained active at the end of 6 months observation period were treated with low dose steroids on the assumption that the skin lesions are manifestation of DHS, even though there was no typical Type I reaction. Two

Table 14(b). Relapse: follow up.

Type	Relapsed months after stopping MDT	Treatment	Results	Follow up
BT	3	No treatment, observation only	Relapsed BB	Died after 2 months (hepatic malignancy)
BT	3		IA residual 7 months	Month, 3 months O.K.
BT	3		IA residual 3 months	8 months O.K.
BT	4		Still active at 6 months observation	
BT	6		Still active at 1 month observation	
BT	6	6 months observation. No improvement. Steroids 3 to 6 months	IA scarred 3 months	4 months O.K.
BT	4	"	IA Residual 6 months	3 months O.K.
BT	5	"	Still on steroids	3rd month improving

responded well, one becoming inactive with scarred lesions and the other with residual lesion. The third is still on steroids and shows improvement at 3 months. It seems that all the cases relapsed with typical Type I reaction, and some of the cases relapsed with active lesions, are due to DHS. So these cases need not be considered as relapsed in the true sense. This means the relapse rate following 6 months MDT is not as high as many of us had feared.

Out of 12 cases which remained active at the end of MDT, 4 never came for follow up. The remaining 8 were observed without treatment. Four became inactive after 3, 4, 8 and 18 months of observation. One remained active at 24 months of observation (facial lesion). Three were put on low dose steroid when they were still active at the end of 6 months' observation. All the 3 became inactive in 3 months and remain inactive after 6, 12 and 14 months of follow up.

Side-effects of drugs used

No side-effects noted. The change in the colour of the urine with rifampicin is actually welcomed by the patients, since many said that the drug was very effective because it can change the colour of urine, or that the disease is brought out through the urine.

In PB cases the MDT actually saves the cost. In MB cases the cost is enormous. It is seventeen times costlier compared with DDS monotherapy for one patient year. However by careful planning and gradual introduction of MDT among MB cases, it is possible that many programmes can introduce MDT without a very high increase in the regular yearly drug budget.

Table 15(c). Follow up of cases which remained active at the end of MDT.

Type	Treatment given	Results		Follow up
BT	No treatment, observation only	IA residual	8 months	14 months O.K.
BT	"	IA residual	18 months	6 months O.K.
BT	"	IA residual	3 months	12 months O.K.
BT	"	IA scarred	4 months	3 months O.K.
BT	"	Still active at (facial lesion)		24 months
BT	Observation 6 months. No improvement. Steroid 3 to 6 months	IA Residual		12 months O.K.
BT	"	IA Scarred		14 months O.K.
BT	"	IA Scarred		6 months O.K.

Table 16. The cost of drugs and patient years saved.

	Patient years saved	Cost saved
Active	3·42	13·06 DM
Inactive	1·72	6·57 DM
Total	2·24	8·55 DM

Cost of 1 year's DDS—3·82 DM. Cost of 6 months' MDT for PB cases—8·38 DM. Cost of one year's MDT for MB cases—65·4 DM.

Workload and caseload

A reduction in the caseload is dramatic, especially in the first two years.

Despite the fall in the caseload the workload remains the same (or) actually increases.

Difficulties faced

The Medical Officer or other responsible person has to visit all the MDT cases once a month to see that the drugs are actually reaching the patient and in order to avoid misuse and abuse of the drugs.

This task is made much more difficult by difficult road conditions, a large area to be covered, 6 months rain, non-availability of petrol, and worsening economic conditions.

Conclusions

1 It seems the course of events in PB cases are mainly determined by the individual's immunological status rather than a particular antileprosy drug or their combination.
2 In the presence of useful immunity in PB cases, the main advantage of MDT over DDS monotherapy seems to be the reduction in the duration of treatment.
3 The fear of large number of relapses following 6 months' MDT seems unjustified.
4 In a situation like ours where only 43·2% of PB cases complete their full treatment and these patients were seen and assessed by the Medical Officer only

once in a year, introduction of MDT has reversed the whole situation in that 80% complete the full course of treatment. All the patients could be seen and assessed every month, with a good 65% follow up.

5 Short-term MDT seems to be the best solution for PB cases in our set up.

Multibacillary cases

Total number of cases analysed, 105; deletions (died, transferred etc.), 8; remaining on treatment, 97; regular (75% attendance), 87 (89·7%); irregular, 10 (10·3%).

The overall attendance rate among BL and LL cases are always higher compared with PB cases since the beginning of the Programme in 1973. However we never once reached 89·7% regular attendance in MB cases. In fact many defaulters and out of control patients whom we could not trace before are returning for treatment, once they have heard about the new drugs.

87 cases (33 BL, 54 LL) who are regular are analysed further

Table 17.

	BL	LL	Total
Active (BACT + VE)	16	30	46
Inactive (BACT − VE)	17	24	41
Total	33	54	87

The 41 inactive cases were put on MDT for the following reasons: 1, They were bacteriologically positive within the last 12 months before MDT started. 2, Most of them needed 10–15 years of DDS maintenance therapy. 3, In the bush clinics, if one patient gets DDS alone and another is given special drugs, it will be considered discriminatory, which might lead to irregularity among DDS monotherapy patients.

Clinical Observation

INACTIVE CASES

As one expected, nothing much is observed in the clinical picture of inactive cases except reactions and other complications which are dealt with later.

Table 18.

Clinical improvement	Very good	Good	No or very little improvement	Total
BL	5 (31·2%)	11 (68·8%)	Nil	16
LL	4 (13·3%)	26 (86·7%)	Nil	30
Total	9	37	Nil	46

ACTIVE CASES

The clinical improvement is graded as: 1, Very good, notable change in the clinical picture within 3 months of MDT. 2, Good, notable change within 6 months of MDT. 3, No improvement, no change at 6 months of MDT.

Table 18 shows that: Percentage wise the improvement is quicker compared to LL (as is true with DDS monotherapy). Comparison of initial BI with clinical improvement showed no definite relationship, in fact the higher the initial BI and more active is the initial infiltration, the more dramatic is the change in clinical picture.

The overall impression is that the clinical improvement is dramatic and quick

Table 19. Twelve (6 BL, 6 LL) out of 46 active cases became bacteriologically negative.

Type	Initial BI	No. of months after MDT became negative
1 BL	1	6
2 BL	1	10
3 BL	2	11
4 BL	2·6	30
5 BL	3·0	17
6 BL	3·5	31
7 LL	1	16
8 LL	1·0	9
9 LL	1·3	19
10 LL	1·3	9
11 LL	1·5	15
12 LL	2·0	24

Average fall in BI in BL cases: 1·46/year.
Average fall in LL cases: 1/year.

with MDT compared to DDS monotherapy but this may be due to the fact that we are observing these patients closely and keenly every month (which we were not doing with DDS monotherapy patients).

Bacteriological observation

There is no relationship between the initial BI and the number of months of MDT needed to make them negative. Fall in BI is quicker in BL (1·46/year) compared to LL (1/year). Analysis of the remaining 34 cases (10 BL, 24 LL) still bacteriologically positive showed that: there is no definite pattern in the fall of BI either in BL or LL; no relationship between the initial BI and the rate of fall in the BI; and MDT seems to have little or no action on the clearance of bacteria as with DDS monotherapy.

Reactions: Type I reaction

Two active cases with the initial BI of 3·4 and 3·3 developed typical Type I reaction and both responded well to steroids. Two inactive cases developed red, well- and ill-defined lesions with very little loss of sensation. These could be called immunological upgrading rather than reaction. Both responded well to continuing MDT.

Table 20. (Four patients, six reactions.)

Type	Month after MDT started	Severity
Active		
BL	3, 11, 26	Moderate
LL	2	Moderate
Inactive		
BL	11	Mild
LL	14	Mild

ENL

Eight out of 87 patients developed erythema nodosum leprosum (rate 9·2%), only two had severe ENL. Varying rates of ENL following MDT has been reported. ENL developing in 42·6% of the patients following MDT has been

Table 21. (Eight patients, ten reactions.)

Type	Months after MDT started	Severity
Known, repeated ENL patients		
LL	9	Mild
LL	17	Mild
LL	9	Mild
LL	1, 6	Mild
Never had ENL before		
BL	4	Mild
LL	14	Mild
LL	23, 26	Severe
LL	9	Severe

reported.[4] The low incidence of ENL in our series seems to be due to the clofazimine we use in our combination. Relief from ENL in repeated ENL patients has restored their confidence in treatment and regularity and also reduced the admissions at the hospital due to ENL.

Drugs—side-effects

No side-effect to DDS or rifampicin were noted. Regarding clofazimine there is a clear change in the colour of the skin. However it is not so obvious as in a fair skinned person.

All the patients accepted becoming a little darker, as long as they could see good clinical improvement and the hope of stopping treatment after some years. Dryness of the skin and conjunctiva was seen in 41·1% and 2·2% of the cases respectively. Strangely, none of them complained about it.

On the day they had to swallow 2 rifampicin + 3 clofazimine + 1 DDS together a few patients complained of vague abdominal discomfort.

Conclusions

1 Early and dramatic clinical improvement is seen.
2 Bacteriological status does not show such improvement.
3 Reduction in the number and severity of ENL attacks, especially in repeated ENL cases.
4 Improvement in our attendance rate to 90%.
5 Drugs are well tolerated and accepted by our patients.

6 We are at a stage when we should start stopping treatment in many cases. However, the uncertainty we had at the beginning of MDT regarding theraputic end-point and the criteria for theraputic end-point still remains.
7 This uncertainty is made worse by the known facts that none of the antileprosy drugs concerned have any significant action on dormant bacilli.
8 In the absence of useful immunity in MB cases, the main use of MDT seems to be the prevention of drug resistance.

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Clinical problems in the initiation and assessment of multidrug therapy

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Introduction

The chemotherapy of leprosy needs both to be as effective and also, certainly under field conditions, as safe, cheap, short, acceptable and easy to apply as possible. Now, 4 years after the introduction of the WHO Study Group regimens[1] is a very appropriate moment to review progress in multidrug therapy (MDT).

The possibility of MDT of limited duration was pioneered by Professor Freerksen and his colleagues in Malta,[2] at the time when the WHO 4th Expert Committee[3] had just extended the recommended duration of dapsone monotherapy in lepromatous leprosy (LL) to '10 years after achieving inactivity', i.e. smear negativity. This entailed a total duration of about 20 years of dapsone in severe LL cases, although with the increasing number of relapses then being seen, many due to dapsone resistance, most leprologists were recommending that LL patients should be treated for life. Borderline–tuberculoid (BT) patients were usually treated for around 4–5 years; the time of achieving clinical inactivity was often difficult to determine and the bacteriological end-point, essential for assessing the effectiveness of chemotherapy, was not differentiated from the ending of immunological activity.

In the 1983 assessment of the small and mixed group of Maltese multibacillary leprosy (MBL) patients (mixed because most had received various durations of dapsone therapy before MDT and their sensitivities to dapsone were not investigated), Jopling[4] found no case of relapse among 116 MBL patients studied 5–108 months after stopping MDT. The significance of the 'solid' bacilli detected in a few smears was uncertain and warranted further investigation, although the ultimate and significant test of chemotherapeutic effectiveness is and will be the relapse rate. Dr Jopling's update on these selected smear positive patients,[5] finding no relapses in April 1986, is therefore of special interest.

The second series of data supporting limited duration treatment has been known for some years, but unfortunately is only now in press. In Malaysia, we

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studied 362 LL and BL patients who commenced treatment as inpatients with sulphones (the majority with dapsone) between 1948 and the end of 1951. Therefore all may be assumed to have been dapsone sensitive. They all received dapsone 300–400 mg twice weekly by injection from around 1950 until 1963 and every dose was recorded. Then many were changed to oral dapsone. All stopped treatment in July 1970. A nine-year follow-up revealed a steady relapse rate of about 1% per annum. Among those relapsed patients who permitted further study, about half were found to have relapsed with various levels of dapsone resistance and half with dapsone sensitive stains of *Mycobacterium leprae*. This suggested that if all drug-resistant mutants could be eliminated by combined chemotherapy, the relapse rate from microbial persistence might well be acceptably low.[6]

In paucibacillary leprosy (PBL), two groups of workers showed that ultra-short course treatment with rifampicin was fully effective. Pattyn[7] with Warndorff in Ethiopia and Bourland in Burundi showed that 8 weekly doses of 900-mg rifampicin cured tuberculoid leprosy; no relapses occurred in a follow-up period of 3 years. In the Philippines, Shepard[8] showed that in tuberculoid (TT) patients given rifampicin 600 mg daily for 14 days and BT patients for 21 days plus, in both groups, two injections of acedapsone, none relapsed during an average follow-up of 2 years, whereas similar groups of patients treated with dapsone for 1 year had a significant relapse rate.

It was on the basis of these four groups of data that the WHO Study Group[1] introduced its recommendations, dividing leprosy patients into two groups, MBL including all LL, BL, and BB patients plus those BT patients with a bacterial index (BI) in smears (when untreated) at any site of 2+ or more, and PBL being TT, BT and indeterminate with no smear site BI (when untreated) greater than 1+. It was assumed that no PBL group patient would have a viable *M. leprae* population greater than 1×10^6 and would therefore be unlikely to harbour rifampicin-resistant mutants.

These recommendations, whether unchanged or with modifications, have now been applied in many parts of the world. In our own practice, and in discussions with many colleagues, we find three major problems equally applicable to Freerksen's and to the WHO regimens.

1 Accuracy of classification

Under field conditions, classification depends on both clinical and bacteriological assessment. Both can be a source of difficulty.

a Bacterial

The sites for slit-skin smears may be ill-chosen. The remedy lies in better training

of those responsible for taking smears. But both adequate privacy and lighting are essential if the best sites are to be detected.

In the laboratory, smear staining must be checked. We have known two good laboratories to experience difficulty, both because of minor misunderstandings over staining techniques, one from under- and the other from over-decolourization. Smear technicians deserve our interest and encouragement. Smear reading and scoring should be standardized.[9]

b Clinical

Patients diagnosed when downgrading from BT towards BL are often difficult to classify as recognizable changes in clinical appearances may not occur until several months after the bacterial load has begun to increase, and the histological classification to shift significantly. The skin smear results, however, should indicate the correct classification of MBL.

Neural leprosy, although nearly always BT or TT, may also occasionally cause problems, as nerve biopsy is seldom possible under field conditions, and even lepromin testing is not routinely performed in most centres. The following brief case histories (full reports to be published elsewhere) of two unusual patients illustrate the difficulties that may be encountered.

Case 1.

A 50-year-old unhappy Bangladeshi lady was referred in February 1980, complaining of numbness. She had been seen frequently by doctors over the previous two years suffering from numerous complaints, but in 1978 had been thought to be anaesthetic over the right hand and wrist. On examination, she was found to have hysterical anaesthesia over most of the body, including the axillae and ante-cubital fossae areas. No skin lesions were visible. But the right superficial radial nerve was significantly though only mildly thickened. She was assumed to have suffered from tuberculoid leprosy in her native country, which had either been treated or which had resolved spontaneously. Biopsy of the enlarged nerve, however, revealed active BL leprosy histologically, and the BI was 5+ (Ridley scale). Skin smears from seven sites (both ear lobes and five random sites) were all negative for acid-fast bacilli (AFB).

Case 2.

This 15-year-old Vietnamese boy was referred in January, 1983. He gave a history of having developed numbness followed by weakness of his right leg and foot some 2 to 3 years earlier. Subsequently he developed numbness of the left leg, and more recently of his fingers. Three months before admission he developed a left foot drop. On examination, no specific skin lesions could be detected. He had

bilateral foot drop, more marked on the right side, and an anaesthetic ulcer was present on the right sole. Most of the nerves of predilection in leprosy were enlarged, and the right ulnar nerve was tender. Skin smears from both ear lobes and four other sites were all negative for AFB. Nerve biopsy (Dr T L Pathi) from the left great auricular nerve area revealed a lymph node whose architecture was destroyed by massive infiltration of activated macrophages, containing 4+ AFB. The nerve was similarly destroyed and in addition to the macrophages or epithelioid cells, there were some lymphocytes. It was considered that although the precise classification was uncertain, the histological diagnosis was 'probably BB, possibly BL'. The Mitsuda reaction measured 3 mm at 4 weeks. Taking all the data into account, he was classified as BB leprosy of the polyneuritic variety.

Under field conditions, both these patients would have been diagnosed as PBL, whereas nerve biopsy revealed that both were in fact MBL, and on WHO criteria should receive treatment for two years with three drugs.

2 Differential diagnosis of relapse and reversal reaction in PBL

Although the word 'relapse' in leprosy can be used in a number of different senses, when antimicrobial chemotherapy is under consideration it signifies the fresh multiplication (and spread) of surviving leprosy bacilli in a patient who had previously responded to therapy. On dapsone monotherapy, such bacteriological relapse was most frequently due to the development of drug resistance, but the MDT regimens have been designed to prevent the selection of drug resistant mutants of *M. leprae*. Of much greater concern now is the possibility of relapse due to the multiplication of persisting, drug-sensitive, physiologically-dormant bacilli occurring after the prescribed course of MDT has been completed.

Relapse in MBL is usually easy to diagnose from the increase in the BI, the finding of new skin lesions (in LL leprosy, often of typical histoid appearance) with a high smear BI containing solid-staining bacilli, and the histological appearances. Bacilli obtained from a new lesion will multiply in the footpads of mice, and the drug sensitivities of the strain of *M. leprae* can be studied.

Relapse in PBL, is much more difficult to diagnose as the appearances often resemble closely, and may be indistinguishable from, those of reversal (upgrading, Jopling type 1) reactions. Indeed, the development of a reversal reaction may occasionally be the first sign of a relapse in BT leprosy. Pandian[10] has recently listed six signs of relapse in PBL, namely the development of: erythema, thickening (infiltration) of skin lesions, new skin lesions, pain and tenderness in nerves, new muscle paralysis, and the extension (increase in diameter) of existing lesions. We have seen all these signs, save the last, in reversal reactions occurring in PBL patients on chemotherapy. But unless records are good, the increase in the size of a skin lesion would probably go undetected—who routinely measures and records the size (diameter) of tuberculoid skin lesions? Moreover, Leiker

(personal communication) considers that extension may very rarely occur in reversal reaction unassociated with relapse. The histological appearances of new skin lesions also may fail to distinguish between relapse and reversal reaction. The development of bacterial positivity, with a rising BI in the smears, is most indicative of relapse provided that chance sampling variation can be excluded, but may not occur in tuberculoid relapse, and if it does, it may not always be detected at a very early stage.

Therefore early relapse may be very difficult if not impossible to distinguish from a mild reversal reaction, unless there is an undoubted increase in the size of the skin lesions, or an undoubted, significant increase in the smear BI. THELEP discussed the timing of the onset of the 'relapse/reaction',[11] suggesting that signs developed within a year of diagnosis, i.e. within 6 months of stopping WHO PBL MDT, were almost certainly due to reversal reaction, whereas signs developed 18 months or more after diagnosis (or 12 months or more after stopping MDT) were more likely to be due to relapse. This could be a useful 'rule of thumb' in alerting clinicians to the possibility of relapse. But reversal reactions can undoubtedly if rarely occur a full three years after commencing effective chemotherapy in BT leprosy.

Case 3.

A 22-year-old East African Asian was seen in consultation in May 1980. He had doubtful very faint hypopigmented macules over much of his limbs. There was widespread enlargement of the nerves of predilection. Smears from six sites were all negative for AFB. Biopsy of the left superficial radial nerve revealed a light infiltrate of lymphocytes but no granuloma cells; AFB were present. He was treated with rifampicin 750 mg daily for 17 days, and with dapsone. After 7 months he developed neuritis, but the then undoubted hypopigmented skin lesions did not develop signs of reversal reaction. He received steroids for 11 months. Throughout 1982, he was prescribed rifampicin 600 mg daily on the first 2 days of each month plus daily dapsone. In March 1983, he developed symptoms of paraesthesiae and anxiety but there was no nerve tenderness. He was given clofazimine and the rifampicin and dapsone were continued. His Mitsuda reaction measured 5 mm at 21 days. In June 1983, 37 months after commencing therapy, during which time his urine was regularly positive for dapsone, he developed an acute right foot drop. On biopsy, the right sural nerve was found to be almost completely destroyed by an epithelioid granuloma, with lymphocytes and fibrosis. Fragmented or granular AFB 1+ were present. The appearances were typical of BT leprosy, which was obviously active, though whether from reaction or relapse was uncertain histologically. In view of his chemotherapy record, and evidence of compliance, there is no doubt that this was a reversal reaction in the nerve.

Although this patient's case is very exceptional in our experience, it poses the

question how long does it take for all granuloma and all AFB to be removed from severely affected BT nerves? A further sural nerve (left) biopsy at 52 months still showed an infiltrate of lymphocytes and fibroblasts in the perineurium and some perivascular lymphocytes within the nerve; although there was no granuloma or definite AFB, scanty AFB granules were still present.

Results to date suggest that short course MDT in PBL is very successful. Personal communications from doctors (Drs E S Thangaraj, P D Samson, A Thomas, L Hogerzeil, G Riedel, J Harris) working in three countries report only 19 'relapses' in over 3800 PBL patients followed for at least one year after stopping MDT; some of these 19 'relapses' may in fact have been reversal reactions. Much longer term follow up is essential, but the outlook appears very promising.

Reversal reaction may also occur late in MBL BL and BB patients. Case 1 developed a swollen right hand with median nerve neuritis, followed shortly by a near complete facial nerve paralysis associated with the development of a hypopigmented lesion (histologically BT) over the upper face and forehead, 56 months after commencing treatment. Under present guidelines she would have stopped treatment at 24 months, but for social reasons plus uncertainty concerning the length of treatment in 'neural' BL, MDT had not been stopped. Had it been, the differential diagnosis of the reversal reaction would have been very difficult especially as the appearance of new BT lesions may be the first sign of bacteriological relapse in dapsone-treated BL patients[12] (Waters & Ridley, unpublished).

3 Histological differentiation of reaction and relapse

Although the histological differentiation of reversal reaction and relapse in skin biopsy is regarded by some workers as difficult, we often find it to be of value. Reaction in mild or incipient cases, that may or may not yet be clinically apparent, is indicated by 1, extracellular oedema in the collagen of the dermis, especially in the neighbourhood of the granuloma, and dilated lymphatics; or 2, a proliferation of fibrocytes (with small dark elongated muclei) throughout the dermis, even far removed from any granuloma. These two features may both be present. In more severe reactions the oedema causes disruption and dispersal of the granuloma in the acute phase. Later there is evidence of upgrading as the granuloma becomes reconstituted (downgrading is not likely to be the cause of a reaction after a period of intensive MDT).

There are 3 possible sources of confusion:

1 Relapse is sometimes associated with upgrading in the new lesion.[12] The relapse lesion simply develops at a point higher up the spectrum than the former lesions, and is not associated with signs of reaction. However, this means that it

may not be possible to distinguish a post-reaction lesion from relapse, and so it is advisable to select the most active-looking lesion for biopsy.

2 Relapse lesions are sometimes highly active, and in such cases there may be a *localized* proliferation of fibrocytes around the periphery of the granuloma. This can be confusing, especially if there is not much normal dermis in the biopsy. A moderate-sized specimen is therefore helpful.

3 In nerves an element of reaction is very common, whether or not there is a generalized rection clinically or histologically, and there is no sharp line of demarcation between activity and reaction.[13] Nerve biopsies, as in case 3, are less helpful therefore for this purpose than skin biopsies.

4 Choice of chemotherapy and its duration

There is no disagreement among scientists either over the principles of MDT or of its objectives, namely to overcome dapsone resistance, to prevent the emergence of new drug resistances, especially to rifampicin, and to cure bacteriologically the great majority of all leprosy patients with acceptable short-course chemotherapy. Nevertheless, there is considerable uncertainty concerning the choice of the third drug to accompany rifampicin and dapsone and over the duration of treatment. Freerksen has preferred prothionamide[2] whereas WHO[1] recommended using the less rapidly bactericidal clofazimine as safer under field conditions, although advising that a thiamide should be prescribed for those patients who rejected clofazimine because of the latter's effect on skin colour.

What is essential today is to assess the toxicity of prothionamide and ethionamide under leprosy control conditions. Among patients using Freerksen's regimen, Jopling[4] has reported one case of jaundice and one case of significantly raised liver transaminases (LFT) in Malta (where monthly estimates of LFT were first introduced in 1979) from 122 MBL patients. Alvarenga *et al.*[14] reported 16 cases of jaundice among 754 patients, both MBL and PBL, receiving Isoprodian (12 of the 16 were also receiving rifampicin) in Paraguay. Much higher incidences of jaundice on combined rifampicin and thioamide treatment have been reported from China[15] and Singapore[16] in which countries clofazimine is not readily accepted, and from France and the French Caribbean.[17,18] Therefore the possibility that genetic and environmental factors influence the incidence of toxic jaundice requires investigation.

In London since 1980 we have treated 37 patients with triple-drug therapy including a thioamide and five have developed jaundice. Two of these were previously untreated lepromatous patients, who commenced treatment in 1980 with rifampicin 600 mg daily plus ethionamide 500 mg and dapsone 100 mg daily. One, a Sudanese, became mildly jaundiced after 28 days, the other, an East African Punjabi, after 12 weeks. The three other patients were long-term dapsone-treated MBL patients who were prescribed a two-year course of

rifampicin 600 mg daily on two consecutive days of each month, ethionamide 250 mg daily and dapsone either 50 mg or 100 mg daily. One was a Cypriot, another an Anglo-Indian and the third a Bengali. Jaundice developed between 2 and $4\frac{1}{2}$ months of commencing triple-drug therapy. In no case was the jaundice severe, and four were subsequently restarted safely on rifampicin, but not on a thioamide. In no case was there any serological evidence of Hepatitis-B virus infection. But two patients did have marginally raised LFT levels before commencing the combined rifampicin–thioamide therapy, and we would now consider the finding of any LFT abnormality, however slight, a contraindication. Only one of the 37 patients studied was Chinese, and one Vietnamese. But in view of the different racial backgrounds of our five jaundiced patients, we have no evidence of a genetic factor. Although this is only a small series, its incidence of toxic hepatitis of 13·5%, very similar to the incidence of 13% reported by Cartel *et al.*,[17] is distressing, and is in marked contrast to that of 1·6% in Malta,[4] of 2·1% in Paraguay,[14] or of 4·5% reported by Pattyn and his colleagues.[19] Further studies appear imperative.

Our limited experience in London does not yet provide any further evidence concerning the duration of treatment required to cure the different types of leprosy; this problem is addressed by other members of the Symposium. Long-term follow-up is essential, with careful investigation of every apparent relapse off treatment, if reliable data are to be obtained.

Conclusions

Although the majority of leprosy patients can be easily allocated to either PBL or to MBL, some may be very difficult, if not impossible, to classify under field control conditions, with resulting uncertainty over the type and duration of treatment required. Good standards of smear taking, staining and reading are essential in all leprosy treatment and control schemes, and scoring of smears should be standardized.

It is often very difficult, if not impossible at least in the short term, to distinguish between reversal reactions and bacteriological relapse off treatment in PBL patients who have completed their course of MDT. All 'relapse' patients require the most careful and thorough investigation if accurate assessment of the results of MDT are to be obtained. The definition of 'relapse' given by the WHO Study Group on the Epidemiology of Leprosy in Relation to Control[20] fails to distinguish between these two groups and is therefore of no help in assessing bacteriological cure rates. Similar failure in the past to distinguish between bacterial relapse and late reversal reaction must cast doubts on many of the previous reports of relapse after completing treatment in tuberculoid leprosy.

Finally, although the basic principles of MDT are generally agreed, we still have insufficient data to decide, on balance, which is the best drug combination

and the duration for which it should be given, to obtain the highest possible cure rates, in the shortest possible time, at the lowest risk of toxic side-effects under leprosy control conditions.

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Preliminary evaluation of the effect of WHO–MDT on disabilities in leprosy patients in Malawi (Central Africa)

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Introduction

Ideally, when introducing a new and supposedly improved treatment, this new regimen should first be tried out in a study group. The results in this study group should be compared with the results in a control group which continued to receive the old treatment. However WHO–MDT was generally introduced without waiting for the results of any such controlled trial on the assumption that (a) multidrug treatment would be superior to dapsone monotherapy, and that (b) there was no time for a controlled trial because resistance of *Mycobacterium leprae* to dapsone was globally reported to be developing into a serious threat to the effectiveness of leprosy control projects.[1] Thus unfortunately it is now only possible to evaluate WHO–MDT using historical controls treated with dapsone monotherapy.

WHO–MDT in paucibacilliferous patients should be evaluated in terms of: a, adverse drug reactions (including death attributable to the regimen); b, development or regression of disabilities; c, Type I reactions; d, relapses; and e, acceptability (compliance and operational feasibility).

All clinical parameters being equal, the cost per patient treated with WHO–MDT should also be compared with the cost per patient treated with dapsone monotherapy.

In this short paper we present a preliminary evaluation of WHO–MDT in paucibacilliferous patients in Malawi in terms of the development or regression of disabilities during and after treatment.

Methods

WHO–MDT in this paper means 600 mg rifampicin supervised intake at intervals

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of four or more weeks, plus 100 mg dapsone daily self-administered (for adults). Completion of treatment means that a patient has taken six supervised doses of rifampicin within nine months of registration.

As a historical control group we chose a previously analysed cohort of patients newly registered in 1975 in the Northern and the Central Region Leprosy Control Projects who, with few exceptions, received dapsone monotherapy.[2] They were generally discharged in accordance with the WHO guidelines in current use before the introduction of WHO–MDT.[2]

The study group consists of paucibacilliferous patients who were newly registered in 1983 in the entire LEPRA Control Project in Malawi. Thus the two groups do not refer to exactly the same parent population.

Treatment for Type-I reactions (delayed hypersensitivity reactions) was similar in both groups and consisted of 30 mg prednisolone daily, reduced by 5 mg every 2 weeks. All treatment was on an outpatient basis.

The disability grading used by the Leprosy Control Assistants in the field was the five grade system recommended by WHO in 1960 and no change has been made since 1973 in the use of this grading system in Malaŵi.[3] The single highest disability grade in a patient (attributable to leprosy) rather than the sum total of all disabilities, was used to determine the level of disability.

Results

The prevention of the development of disabilities is generally stated as one of the main objectives of a leprosy control project[4] and it therefore seems relevant to show in particular which percentage of newly registered patients developed new or higher levels of disabilities during or after completion of treatment.

Table 1 shows that of 831 new paucibacilliferous patients registered in 1983 and reviewed after completion of treatment, 31 patients (3·7%) either developed new disabilities (having grade 0 at registration) or that their disabilities worsened during WHO–MDT. So far, data are available for 545 patients who were due and available for review examination one year after completion of WHO–MDT. The percentage of patients with newly developed or worse disabilities since registration had increased to 5·7% by that time. Table 1 also shows that in 50% of those with disabilities at registration the level of disability decreased during and after WHO–MDT. The majority of these (57/75) regained sensitivity in hands or feet which had been anaesthetic at registration.

In the control group of 264 tuberculoid and borderline leprosy patients in the Northern Region and 262 patients in the Central Region Leprosy Control Projects review notes are only available for the point in time of discharge, since these individuals were not kept under active surveillance after completion of treatment. The review notes at discharge indicated that seven patients (2·7%) in the Northern and 16 patients (6·1%) in the Central Region had developed new

Table 1. Historical comparison of the development of disabilities during and after WHO–MDT with the development of disabilities during dapsone monotherapy in leprosy patients in Malawi (Central Africa).

	Control groups		Study group
	Northern Region LCP Tuberculoid and borderline leprosy, 1975 cohort	Central Region LCP Tuberculoid and borderline leprosy, 1975 cohort	LEPRA Control Project Malawi Paucibacilliferous patients registered in 1983
Number of patients registered	380	479	862
Percentage of patients with any disabilities at registration (WHO grading 1960, = or > 1) and with complete review notes at 'discharge' = completion of treatment	10·6% (29/264)	19·5% (51/262)	17·3% (144/831)
Percentage of patients with worse disabilities at 'discharge' (= completion of treatment) than at registration	2·7% (7/264)	6·1% (16/262)	3·7% (31*/831)
Percentage of patients with worse disabilities one year after 'discharge' (= completion of treatment) than at registration	—	—	5·7% (31*/545)
Percentage of patients with less disabilities at 'discharge' than at registration	27·6% (8/29)	19·6% (10/51)	52·1% (75/144)
Percentage of patients with less disabilities one year after 'discharge' than at registration	—	—	51·7% (46/89)

* not all the same individuals

disabilities during treatment or were found at discharge with disabilities worse than at registration. This range of 2·7–6·1% is likely to be representative for the outcome after many years of dapsone monotherapy in Malawi. In this control group eight out of 29 patients (27·6%) in the Northern and 10 out of 51 patients (19·6%) in the Central Region were found at discharge with a decrease in disabilities.

Discussion

Our comparison of the 1975 with the 1983 cohort in terms of disabilities would seem to be reasonably valid, because the organizational structure, diagnostic procedures and the assessment of disabilities did not change appreciably between 1975 and 1985 within the LEPRA Control Projects in Malaŵi. One indication for the comparability of the two groups is the similarity of the level of disability at registration which is particularly similar in the study group and the 1975 Central Region cohort of newly registered patients.

On the other hand there is only a partial overlap of the recruitment areas of the two groups and the 1975 cohort includes some patients with borderline leprosy, who, if registered in 1983, would perhaps not have been classified as paucibacilliferous patients. In addition, treatment for Type I reactions was probably more vigorously instituted in the eighties than in the seventies.[2] This last difference would lead one to expect a slightly better outcome in those patients registered in 1983 than in those registered in 1975.

Nevertheless our preliminary analysis presented here shows that the percentage of patients who developed new disabilities or experienced a deterioration in disabilities during or after WHO–MDT was within the range of such adverse outcomes seen in patients treated with dapsone monotherapy.

In fact, the outcome one year after completion of WHO–MDT (5·7% with new or worse disabilities) is remarkably similar to the outcome in the Central Region 1975 cohort which was unfavourable in 6·1% of patients at the end of at least 3 years dapsone monotherapy.

There seems to be some evidence however that a higher percentage of patients with potentially reversible disabilities improved during or after WHO–MDT than after dapsone monotherapy. One could speculate that this might be due to a swifter action of multidrug treatment in arresting the disease process and thus facilitating recovery of nerve function.

This aspect might warrant an investigation in a controlled trial since the methodology of using historical controls as in this analysis is an obviously crude and potentially misleading one.

Acknowledgment

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The use of MDT in the three Western Regions of Nepal

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The Leprosy Control Programme in West Nepal

The Leprosy Control Programme (LCP) covers 21 districts in the three western regions of Nepal, and provides treatment for more than 10,000 leprosy patients in about 200 treatment units of the Basic Health Service, and several hospitals/ referral centres. PMWs work at various levels, visiting health posts and running clinics on a pre-fixed date. The number of clinic days per month varies according to the number of patients per unit, but in most of the treatment places there are less than 25 registered patients, and those places are attended bi-monthly.

The majority of treatment units are not easily accessible, and most of the travelling has to be done on foot, which considerably limits the supervisory assistance given to the paramedical staff and consequently the PWM must be able to deal with the entire care of leprosy patients, including diagnosis, dealing with complications, and release from control.

Introduction of MDT

The WHO recommended regime of MDT was introduced to the LCP in Nepal in April 1982, following the second National Workshop on Leprosy Control in November 1981 in Kathmandu. Guidelines for the country were defined at that time.

In the three western regions of Nepal, MDT is now used in eight districts and in three specialized referral centres. (General information on the number of patients treated in the programme in West Nepal, as well as those who have been or are on MDT, is given in Table 1.)

The introduction of MDT to the operational pattern of the LCP was assisted by the following measures:

A standing order was designed (in English and the national language, Nepali),

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which gave the PWM all the necessary details for handling MDT on their own.

The necessary structured training was given to paramedicals.

The recording and reporting system of the programme was adjusted to MDT and necessary additions were made.

The monthly supply of medicine was packed in convenient plastic bags (this assists the pattern that one dosage of rifampicin and 300 mg clofazimine can be taken unsupervised).

A leaflet was designed for the patient giving him basic information on MDT and instructions on how to take the medicine.

A poster was produced to assist the health education campaign.

Area of evaluation

To evaluate the operational set-up of MDT, four studies were performed, one in a referral centre and three in the field.

Green Pastures Leprosy Hospital[1], where MDT was started first (in April 1982), is an old established place with about 1000 outpatients and acts as a referral centre. Outpatients are routinely seen by the PWM staff only. Seven months later a study was done, investigating the functioning of the operational set-up of those patients coming from one district only (Kaski District, surrounding the hospital).

In the same district, Kaski, leprosy patients are also treated in 12 health posts according to the described operational set-up of the LCP. One year after MDT was introduced into those health posts the operational set-up was investigated as well.

In the Western Terai, MDT was started in the Banke District at the same time as in Kaski. Many patients are treated there. The operational set-up in the health

Table 1. Patients on multidrug therapy

	Field			Referral centre			Total		
	Multi	Pauci	Total	Multi	Pauci	Total	Multi	Pauci	Total
No. of patients on MDT	383	566	949	575	257	832	958	823	1781
Patients already released from MDT	—	306		—	268		—	574	

Total No. of patients: field, 5863; referral centre, 4740. Total 10603
Twenty-one per cent of patients are receiving or have received MDT.

post of the district capital, Nepalganj, treating 456 patients, was also investigated one year after MDT was introduced.

In addition to this, the study in the Banke District has been extended to four more health posts to give additional information beyond the health post in the district centre, Nepalganj.

The whole study was concluded in mid-1983.

Results

NUMBER OF PATIENTS ON MDT

About one year after MDT was introduced, about 80% of all registered patients were on MDT (Table 2).

OPERATIONAL SET-UP

Detailed measures in relation to the operational set-up of MDT as described above were only done after MDT had already been started in the Green Pastures Leprosy Hospital. For this reason, this hospital basically continued handling the patients according to the previous regime. Most of the faults found were, therefore, related to lack of clear operational instructions.

Clinical examination performed at the start of MDT (Table 3)

In both field areas, the Kaski District as well as the Nepalganj health post in the

Table 2. General information

Treatment unit	No. of patients registered	No. of patients on MDT			% of patients on MDT
		Multi	Pauci	Total	
GP referral centre patients from Kaski District only	350	129	126	255	(73)
Kaski District 12 HP	155	35	89	124	(80)
Banke District Nepalganj HP	456	135	242	377	(82)
Banke District 4 HP	178	45	113	158	(89)

Table 3. Operational set-up. Examination performed at the start of MDT

Treatment unit	Clinical examinations No.	(%)	Smears taken No.	(%)
Kaski District only GP referral centre	?	?	133	(52)
Kaski District 12 HP	123	(99·2)	119	(96)
Banke District Nepalganj HP	367	(97·3)	326*	(86)

* Twenty-six smears which were taken later or earlier are not included.

Banke District, almost all patients were examined at the beginning of MDT, while those figures were not evaluated at the Green Pastures Leprosy Hospital.

Bacteriological examination at the beginning of MDT (Table 3)

As far as smear-taking is concerned, it becomes particularly obvious that the lack of clear instructions when MDT was introduced to the Green Pastures Leprosy Hospital resulted in an insufficient number of smears being taken at the beginning of MDT (but it must also be stated that a number of smears had been taken in recent months before the patient was put on MDT). In both field areas, the number of smears taken is sufficiently high, although in the Banke district not all were taken exactly at the right date.

Table 4. Operational set-up. Smear results recorded in patients' records at beginning of MDT (including some smears taken later or earlier)

Treatment unit	No. of smears taken	Smears not recorded No.	(%)
Banke District Nepalganj HP	345	40	(11·6)
Banke District 4 other HP	157	50	(32)
Kaski District 12 HP	119	9	(7·6)

Table 5. Operational set-up. Monthly unrecorded smear results outstanding

	1/12	2/12	4/12	5–6/12	11/12
1 of the 4 HP Banke District	—	—	1	4	10 (!)
Kaski District					
12 HP	6	3	—	—	

A smear result should be reported after 2/12, but not after 4/12.

Recording of smears performed (Tables 4 and 5)

Smears are taken in the field, fixed and despatched to the referral centre concerned via the control office or, in some cases, to a laboratory of the Basic Health Services. The reports from referral centres are communicated via the office to the centre of each district; from there the PMW takes them to the different health posts.

This administrative line is rather complicated and liable to failures. Evaluating the results of Kaski district first, 7·6% of smears were outstanding, but only for 1 to 2 months (Table 5). Concerning the 'smear chain' described above, this is normal, and so it can be said that in Kaski District no smear result was lost.

This is particularly different in the four other health posts investigated in the Banke District. A detailed study of one of these health posts revealed that the majority of smears are already 11 months late! On investigation, it became obvious that the fault is mainly in the field: smears are despatched too late to the office; smear results are kept in the district centre only, and are not taken to the individual health posts; and there was a decrease in the work performance since the introduction of MDT (compare Nepalganj health post with the four others in Banke, Table 4).

Evaluating some factors of the operational set-up emphasizes that:

The operational set-up must be clear before MDT is started and detailed instructions must be given to the PMW staff to enable them to handle their work with confidence. This applies equally to the field and to hospitals, even if more expert personnel is available in the latter.

After adequate training, the staff need to be sufficiently introduced to their duties and periodically supervised in areas where they work on their own.

QUALITY OF CLINICAL WORK

Clinical examination activity or inactivity (Table 6) *and recognition of complications* (Table 7)

The result of the field-work in both districts shows great uncertainty of the PMWs

Table 6. Quality of clinical work. Clinical examinations: patients active or inactive at the start of MDT.

Multibacillary	Active	Inactive	No record of or records disagree (%)
Kaski	11	4	20(57)
Nepalganj HP	47	43	42(32)
Paucibacillary			
Kaski	—	41	48(54)
Nepalganj HP	57	171	7(3)

concerning the decision of whether a patient is active or inactive. Examination of records reveals that active symptoms are listed, but the patient in fact is considered as inactive; or records are incomplete on this issue. Frequently it is also difficult for the PMWs to make decisions because previous records are incomplete.

In a similar way, complications are insufficiently recognized and recorded. It is unlikely that the number of reactions is below 5% after MDT is introduced.

Drug side-effects are mostly not recorded at all.

The uncertainty in reaching proper conclusions after clinical examinations and not recognizing complications in time applies also to good paramedical staff. The findings, on the one hand, emphasize the need for sufficient training, particularly with clinical practice and constant re-training and, on the other, for a recording system which reminds the PMW to follow the proper examination pattern in order to come to the right conclusions.

We have recently developed a new patient recording system which follows far more the pattern of ticking-off certain symptoms. It leads automatically also to those in relation to complications and, in cases where they are present, the PMW is asked to fill in a special Complications Form. In this way we hope to overcome such weaknesses revealed in the current study.

FOLLOW UP

Regularity of patients before and after starting MDT (Table 8)

It is interesting to see that, in fact, the regularity under MDT has only gone up in the health posts of the Kaski District and of the Green Pastures Leprosy Hospital. There was a drop at the Nepalganj health post, and particularly at the other four

Table 7. Quality of clinical work. Complications recorded.

	Reactions
	No. (%)
Green Pastures	6(2·4)
Kaski	4(3·2)
Nepalganj	19(5)

Drug side-effects—records are too unclear to draw conclusions.

Table 8. Regularity. Definition of MDT regularity: Number of once-monthly doses given and once-monthly doses possible (%)

	Regular on MDT		Regular before MDT	
Treatment unit	No.	(%)	No.	(%)
Green Pastures	192	89	327	83
Kaski District				
12 HP	102	85	119	74
Nepalganj	255	73	321	84
Banke District				
4 HP	104	66	127*	79

* 3 HP only

health posts of the same district, Banke. In fact for some months the work performance decreased after MDT had been introduced.

The routine system introduced to follow up late patients is: a registered letter is written after the patient has failed to attend a clinic day; and if the patient fails to attend again, a home visit is done.

Other studies in the programme have shown that there is a response to letters of about 30%, while there is even less to home visits. Home visits are popular amongst the PMW staff because they get extra travelling allowance for these, while letters are just additional work. The latest supervision visit revealed that letters had not been sent for 8 months in the Banke District. Home visits had to be

Table 9. Regularity. Compliance. Clofazimine skin discolouration

	Present	Absent No. (%)
Green Pastures	84	2(2·4)

stopped because of a budget shortage. The effect of these can be questioned anyway. The situation needs careful investigation to make sure that the regularity of attendance will reach an acceptable standard again.

Compliance (Table 9)

This study was done in Green Pastures Leprosy Hospital only, but it showed that almost all patients put on clofazimine were, in fact, taking the medicine. This was also confirmed by the field staff of the Kaski District.

Frequency of once-monthly dosage missed (Table 10)

Clinic days in health posts attended by the staff of the LCP are fixed, and in many places are bi-monthly only. If the patient comes on the wrong day, he will get antileprosy treatment but no rifampicin. But the patient must take his minimum dosage of rifampicin. If they miss it in certain months, this time needs to be added to their whole treatment period. For example, if a patient on paucibacillary treatment misses it for 4 months, his whole treatment period will be 10 months.

The evaluation shows that in those four health posts in the Banke district less than 50% of the patients never missed a clinic day and received all their monthly doses of rifampicin continuously. More worrying are those patients who missed more than 4 times their monthly dosage (up to 31%).

Table 10. Frequency of once monthly dosage missed

	None		Up to 3/12		4/12–6/12		More than 6/12	
Banke District 4 HP	No	(%)	No	(%)	No	(%)	No	(%)
Multibacillary	12	(27)	17	(38)	8	(18)	8	(18)
Paucibacillary	57	(50)	23	(20)	12	(11)	21	(19)
Total	69	(44)	40	(25)	20	(13)	29	(18)

Conclusions

It has been proved that, even under very difficult field conditions like those in Nepal, MDT can be introduced in the field if there are detailed instructions available for the PMW, together with sufficient training. In addition, supervision needs to be done, particularly at the beginning to introduce the new system properly, as well as periodically afterwards to make sure the system is followed. We are hoping that the new patient recording system will help to improve the standard further, and particularly to allow us precise evaluation of the programme in the future.

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Operational aspects of the implementation of multidrug therapy at ALERT, Ethiopia

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Introduction

The ALERT Leprosy Control Department is responsible for leprosy control in Shoa Administrative Region (Figure 1). This region is centrally located in Ethiopia; it covers an area of about 85,000 sq km, with a population of 8·75 million. The region is divided into one urban and 11 rural districts. Leprosy diagnostic and treatment services are given in 292 centres, 176 (60%) are attached to the general medical services and 116 (40%) are leprosy clinics, which have been established in those areas where a general medical service does not exist yet. About 50% of the centres are accessible by car during the whole year; 32% are accessible by car during the dry season only, while 18% are not accessible by car.[1]

Multidrug therapy (MDT) in the ALERT Leprosy Control Programme

Multidrug therapy (MDT) according to the recommendations given by the World Health Organization (WHO) in 1982,[2] was introduced in the ALERT Leprosy Control Programme in January 1983. Paucibacillary (PB) patients are treated for a period of 6 months, with dapsone daily self-administered and rifampicin monthly under supervision. Multibacillary (MB) patients are treated for a period of at least two years, and until the skin smears (Bacteriological Index) have become negative, with daily dapsone and clofazimine self-administered, and monthly rifampicin and clofazimine under supervision.[3]

During 1983 MDT was introduced into two districts, Tegulet & Bulga and Yifat & Timuga, in the north-eastern part of the region, the Debre Berhan area, including 64 clinics. During 1984 MDT was extended to three districts, Addis Ababa, Menagesha and Yerer & Kereyu, in the central part of the region, the Addis Ababa area, including 48 clinics.

In December 1985 MDT was extended to two further districts, Haykoch and Butajira and Kembata & Hadiya, in the southern part of the region, the Southern

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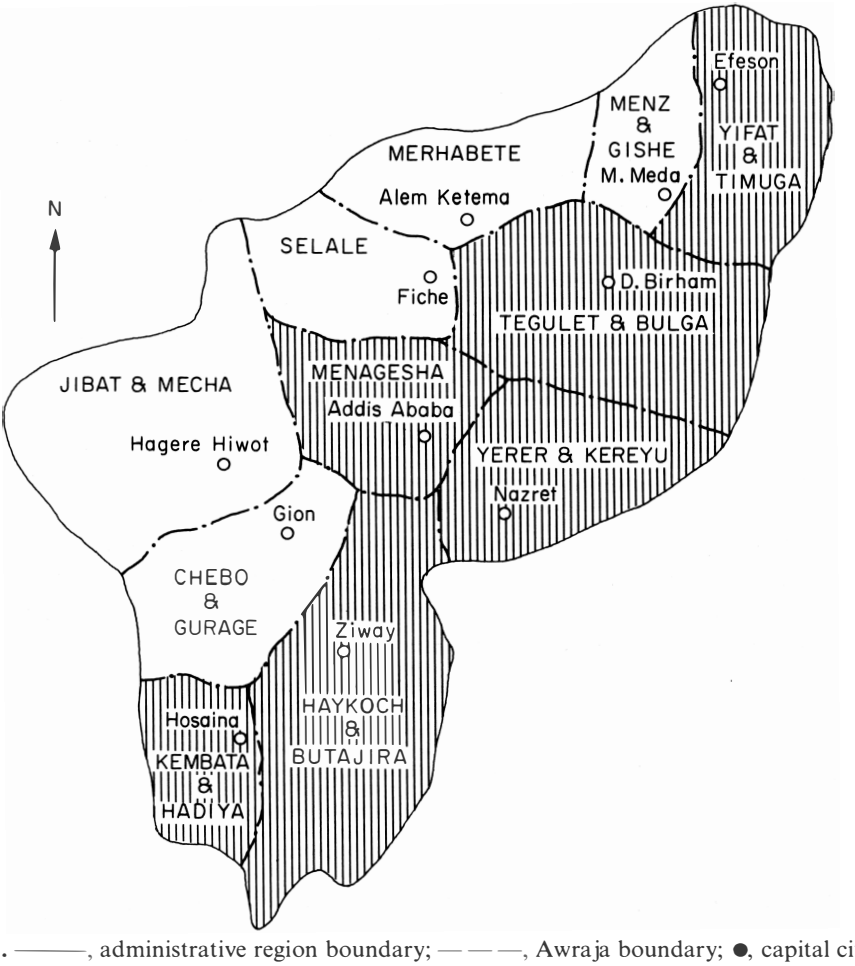

Figure 1. ———, administrative region boundary; — — —, Awraja boundary; ●, capital city; ○, Awraja capital; ///, MDT area.

Shoa area, including 61 clinics. During the last quarter of 1986 two more districts, Selale and Menz & Gishe, will be included in the MDT programme, while the last three districts of the region, Merhabete, Gibat & Mecha and Chebo & Gurage, will be included in the MDT programme during the period 1987–1989 (Figure 1).

Prior to the introduction of MDT the leprosy control services are reorganized and intensified. This includes clinical and bacteriological examination of the patients under treatment, (re)assessment of the classification, and release from treatment of those patients who are considered to have received sufficient chemotherapy with dapsone monotherapy. Furthermore, new recording and reporting systems are introduced, health education is given to patients and the communities, and the tasks and training of all cadres of staff are re-defined.

By January 1986, 7587 patients (3320 PB and 4267 MB) had been put under MDT.

Release from treatment prior to introduction of MDT

During the period January 1983–January 1986 6005 patients (4186 PB, 1769 MB and 50 unclassified borderline) have been released from dapsone monotherapy. These patients fulfilled the criteria of regular attendance of at least 5 years for PB patients and at least 10 years for MB patients and of clinical and bacteriological inactivity.

In the Addis Ababa area 218 TT, 685 BT and 1005 BL and LL patients were released from dapsone monotherapy during the period July 1983–July 1984.

Among the BT patients five relapses have been diagnosed; among the BL and LL patients, 57 relapses. The relapse rate in the BT patients is 7·3/1000 in about two years which is 3·65/1000 per year. The relapse rate in BL and LL patients is 57/1000, which is 28·5/1000 per year. Data about the period between release from treatment and diagnosis of relapse is available on the patient record cards and will be analysed when our programme is computerized.

All relapsed patients presented themselves at our clinics. Active tracing of patients who do not come for regular examinations after release from treatment is not carried out. Therefore, it is not known whether these relapses represent only a proportion of the real number of relapses.

Results of implementation of MDT

THE DEBRE BERHAN AREA

In the Debre Berhan area 3509 patients were under chemotherapy by January 1983. Prior to the introduction of MDT only 26 PB patients were released from treatment (RFT). Due to incomplete recording of signs of inactivity it appeared not to be possible to apply guidelines defined by the WHO for release from treatment,[4] while at the time of introduction of MDT instructions for RFT of patients in Ethiopia had not been defined. During the first months of 1983, 3140 patients were put under MDT. The remaining 369 patients continued dapsone monotherapy because they were either not able or willing to attend monthly for the supervised treatment or had attended very irregularly. Results of completion of treatment are evaluated through cohort analysis. The results of the four six-monthly cohorts of PB patients for 1983 and 1984 are given in Table 1.

Of the 1944 PB patients who started MDT during 1983 and 1984 1715 patients (88·2%) completed their course of MDT and were released from treatment; 188 patients (9·7%) discontinued the treatment. Of the remaining 41 patients (2·1%) 12 died, 17 were transferred out and 12 continued the treatment after the 9 months which are allowed for completion of the course of MDT. These results are considered quite satisfactory. Compared with 1983 a decrease in the proportion

Table 1. Results of completion of MDT of PB patients: Debre Berhan Area

	Cohort 1 1983		Cohort 2 1983		Cohort 1 1984		Cohort 2 1984		Total	
	No. patients	(%)	No. patients	(%)	No. patients	(%)	No. patients	(%)	No. patients	(%)
Put under MDT	1684	—	70	—	130	—	60	—	1944	—
Released from MDT	1501	(89·1)	64	(91·4)	107	(82·3)	43	(7·7)	1715	(88·2)
MDT discontinued	157	(9·3)	5	(7·1)	18	(13·9)	8	(13·3)	188	(9·7)
Died	12	(0·7)	—	—	—	—	—	—	12	(0·6)
Transferred out	14	(0·8)	1	(1·4)	1	(0·7)	1	(1·7)	17	(0·9)
Continued MDT	—	—	—	—	4	(3·1)	8	(13·3)	12	(0·6)

Table 2. MB patients struck off registers

	No. patients (%)
Released from MDT	802 (71·3)
MDT discontinued	120 (10·7)
Died	68 (6·0)
Transferred out	124 (11·0)
Left area without transfer	10 (0·9)
Total	1124 (99·9)

of patients who completed their course of MDT can be observed for the patients who started MDT during 1984, while the proportion of patients who discontinued the treatment increased. The severe drought situation which has affected large parts of the area since the end of 1984 has been identified as the main reason for these results.

The first cohort of MB patients for 1983 will be evaluated by September 1986.

During the period January 1983–January 1986 1124 MB patients have been struck off the registers, for reasons given in Table 2. The majority of the 120 patients who discontinued the treatment had attended regularly during 1983 and 1984, had received dapsone prior to MDT and had negative skin smears before the start of MDT or after 1 year of MDT. Therefore many of them may be considered to have received sufficient chemotherapy; however, as they were not formally released from treatment after clinical and bacteriological assessment, they are considered not to have fulfilled our criteria for release from treatment.[3] The main reason for discontinuation of the treatment has been the severe drought situation. This also applies to the high number of deaths. Due to resettlement of large groups of the population the number of patients who were transferred is high.

Until January 1986 one BT relapse has been diagnosed in a patient who did not receive dapsone prior to MDT.

By January 1986 2268 patients were reported to be under chemotherapy, 1877 patients under MDT and 391 patients under dapsone monotherapy. In this area the number of patients under chemotherapy has been reduced to 56% within a period of $2\frac{1}{2}$ years.

THE ADDIS ABABA AREA

In the Addis Ababa area 1952 patients were released from chemotherapy prior to introduction of MDT. From March 1984 to January 1986 2744 patients, 1861 MB and 883 PB patients, have been put under MDT. Results of completion of treatment of the two six-monthly cohorts of PB patients for 1984 are given in

Table 3. Of the 494 patients who started MDT during 1984 484 (98·0%) completed their course of treatment; a very satisfactory result. By January 1986 no MB patients had been released from MDT. Over 90% of the MB patients attended regularly for their treatment.

Compliance studies, by way of the urine spot test for the presence of dapsone, are carried out with the PB patients during the 4th and 6th supervised treatment round and with the MB patients during the 4th, 6th, 12th, 18th and 24th treatment round. The results for the fourth up to the 18th treatment round are given in Table 4. The results are encouraging, especially when compared with results of previous compliance studies carried out at ALERT, which gave 60–70% positive urines.

The decline in proportion of positive urines, especially during the eighteenth treatment round, could indicate a decline in motivation of patients for the new treatment.

By January 1986 2268 patients were reported to be under chemotherapy, 1877 patients under MDT and 391 patients under dapsone monotherapy. In this

Table 3. Results of completion of treatment of PB patients: Addis Ababa Area

	Cohort 1 1984		Cohort 2 1984		Total	
	No. patients	(%)	No. patients	(%)	No. patients	(%)
Put under MDT	386	—	108	—	494	—
Released from MDT	382	(99·0)	102	(94·4)	484	(98·0)
MDT discontinued	3	(0·8)	5	(4·6)	8	(1·6)
Died	—	—	—	—	—	—
Transferred out	1	(0·2)	1	(0·9)	2	(0·4)
Continued MDT	—	—	—	—	—	—

Table 4. Results of urine spot test during different treatment rounds

	Urines tested	positive (%)	negative (%)
4th round	1311	1194(91·0)	117(8·9)
6th round	1284	1101(85·8)	183(14·3)
12th round	927	795(85·8)	132(14·2)
18th round	1002	768(76·6)	234(23·4)
Total	4524	3858(85·3)	666(14·7)

area the number of patients under chemotherapy has been reduced to 56% within a period of $2\frac{1}{2}$ years.

THE SOUTHERN SHOA AREA

In the Southern Shoa area 2104 patients were released from treatment prior to introduction of MDT.

In the two districts the urine spot test for the presence of dapsone was carried out on a sample of patients who had been selected for MDT, prior to their start of MDT. Out of the 649 urines which were tested 438 were positive (67·5%). The result is similar to that obtained at ALERT during the dapsone monotherapy era. Compliance data on the self-administration of dapsone before implementation of MDT will be compared with those after MDT has been introduced.

By January 1986, one month after the start of implementation of MDT, 1190 patients, 732 MB and 458 PB patients, had started their course of MDT, while 1157 patients were still under dapsone monotherapy.

Prospects for leprosy control in the ALERT Leprosy Control Programme

During the period July 1982–July 1985 the number of patients under chemotherapy, MDT and dapsone monotherapy, in Shoa Region, decreased from 20,908 to 10,507; a reduction of 50% within a period of 3 years. This decrease is due to the application of instructions for release from treatment of patients after monotherapy[3] and the introduction of MDT.

By 1993, 3 years after the last district of Shoa Region will have been included in the MDT programme, all but a few of the patients under chemotherapy, will be newly diagnosed patients and patients who have relapsed, who hopefully will be few.

Assuming that there will not be major changes in the number of newly diagnosed patients in the region, the number of patients under chemotherapy will, from 1993 onwards, be in the order of 2000–3000 at any time. This is a reduction of 85–90% in the number of patients under antileprosy treatment within a period of 10 years. With 292 leprosy diagnostic and treatment centres in the region, the average number of patients per clinic will then be from seven to ten. If the decline in detection of new patients, which has been observed during the last few years, continues, the number of patients under chemotherapy will be even less. Although the workload, related to the number of patients under chemotherapy, will gradually decrease, the total workload will continue to remain high for many years due to:

1 Provision of care directed at existing and potential increase of disabilities. In the ALERT Leprosy Control Programme about 35% of the known leprosy

patients are in need of regular care for existing disabilities or threatening increased disability. If the trend in steady, although slow, decrease in the proportion of new patients who have already severe disabilities at the time of diagnosis, a trend which has been observed during the last few years, continues, and if reactions are diagnosed early and properly treated and continuous care can be given to prevention of increase of disability, this group of patients will gradually decrease. However this decrease is not expected to be very fast. The figure for 1985 of 18% of new patients who had already a disability grade 2 or 3 at the time of diagnosis of leprosy is still high. Our method of case-detection has almost exclusively been passive over the years. A more active detection of patients, an approach which has been formulated as one of our priorities, whenever feasible, could lead to a steeper decline in the proportion of new patients with severe disability.

2 The need for regular follow-up examinations of patients who have been released from MDT. So far about 40% of the patients have attended for the appointed follow-up examinations. With a follow-up period of 5 years after release from treatment, the group of patients who need to come for follow-up examinations will gradually decrease. In the ALERT Leprosy Control Programme this group will, from 1998 onwards, comprise mainly newly diagnosed patients.

3 Another matter for serious concern is the integration of leprosy and tuberculosis control activities. The leprosy control infrastructure could provide a sound basis for extension of tuberculosis control in the rural areas (1·5).

MDT field evaluation studies

It is planned to carry out three MDT field evaluation studies in 41 out of the 48 clinics of the Addis Ababa MDT area.

These studies concern: frequency of occurrence of relapses after treatment; frequency of occurrence of leprosy reactions during and after treatment; and nerve function impairment during and after treatment.

The leprosy control activities in the 41 study clinics will remain basically the same as in all other clinics where MDT has been implemented in the ALERT Leprosy Control Programme.

However, there are some differences:

1 For MB patients the duration of the MDT course is limited to 2 years, while in the routine services the treatment is continued till skin smear (Bacteriological Index) negativity.

2 The surveillance of patients after the chemotherapy course is 'active' in the study clinics, while 'passive' in the routine service clinics.

3 The study clinics will generally have more intensive supervision and for

quality control some additional assessments are done, e.g. urine spot test for dapsone intake compliance.

Problems encountered with implementation of MDT

The following problems were encountered during the implementation of MDT:

1 The severe drought situation

Large parts of the Debre Berhan area were affected by the severe drought. This has caused a decline in attendance by MB patients and by a high number of patients who were, because of resettlement, transferred to a non-MDT area.

2 Acute shortages of public transport

Because many of the public buses were used in resettlement programmes, we experienced acute shortages of public transport. Field staff had to rely on alternative ways of transportation, and often they had to walk to the clinics.

3 Analysis of data

The amount of paperwork has substantially increased. Compilation of analysis 'by hand' of the many data which are essential for evaluation of MDT and the leprosy situation over the years have become very time consuming tasks. At present much of the available data remains unused.

4 Differentiation between relapse and reversal reaction after release from treatment

So far there are no criteria for distinction between relapse and reversal reaction after release from treatment. During the first months of 1986 six BT patients from the Debre Berhan MDT area were diagnosed with a reactivation of the disease with involvement of nerves, 12 months or more after their release from MDT.

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Combined chemotherapy of multibacillary leprosy of 6 months' duration

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Introduction

It has been shown previously[1] that a regimen composed of rifampicin (RMP) 600 mg twice a week together with daily prothionamide (PRO) 500 mg and dapsone (DDS) 100 mg given during 6 months followed by 6 months of daily 100 mg DDS was effective in the treatment of multibacillary leprosy.

Since dapsone (DDS) is essentially bacteriostatic and slowly bactericidal for *Mycobacterium leprae*, the role of the dapsone monotherapy during the second semester in the above regimen may be questioned. It was therefore justifiable to explore the value of a 6 months' regimen.

Patients and procedures

Untreated multibacillary (MB) patients and relapsing, active MB patients were included in the study. Routine clinical and neurological examinations were performed; smears from one earlobe and two skin sites were examined for acid-fast bacilli. A skin biopsy was taken for histopathology. The following supervised treatment regimen was given: Regimen RPD—2 weeks RMP 600 mg 7/7, PRO 500 mg 7/7, DDS 100 mg 7/7, followed by 24 weeks RMP 600 mg 1/7, PRO 500 mg 7/7, DDS 100 mg 7/7.

Old MB cases treated for 5 years or more with DDS—and thus at risk of being infected with DDS-resistant organisms—were given instead clofazimine (CLO): regimen RPC.

The patients were seen regularly and formal clinical, neurological, bacteriological and histopathological examinations were performed at yearly intervals.

Results and Discussion

Between early 1982 and early 1984, 74 patients were taken into the trial, 54 males

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(73%) and 20 females (27%). The age distribution is presented in Table 1; 50% are 45 or more years old. Forty-seven patients had not been treated before; 27 were old cases clinically active having been treated for various lengths of time with DDS monotherapy, sometimes very irregularly; four of them had taken DDS for less than 5 years and thus received the regimen RPD. One new patient was erroneously treated with the combination RPC.

The mean bacterial index (BI) was 4·05. Many patients were lost because they were transferred to another centre where dapsone was continued because they still had a positive BI.

Because of the comparable antibacterial activity of DDS and CLO, the

Table 1. Age distribution of patients taken into the trial.

0–5	1
6–15	3
16–25	5
26–35	10
36–45	18
45+	37

Table 2. Follow-up of patients taken into the trial.

	NC		OC		Total
	RPD	RPC	RPD	RPC	
Total taken in	46	1	4	23	74
Died	2			2	4
Lost	18	1	3	13	35
Analysable	26	—	1	8	35
1Y	3			3	6
2Y	15		1	2	18
3Y	8			3	11

NC, new cases; OC, old cases; RED, rifampicin, ethionamide, dapsone; REC, rifampicin, ethionamide, clofazimine

patients who received RED and those who received REC may be considered together.

In total 29 patients (17 males (58%) and 12 females) were followed for 2–3 years, when the mean BI had dropped to 1·8. No relapses were observed. The hepatitis cases mentioned in a previous publication[2] occurred in patients who were not followed for two or more years. The observation continues.

The only criterion allowing the assessment of therapeutic regimens in MB leprosy is the eventual appearance of relapses.[3] It has been found[4] that 50% of relapses in MB leprosy occur within the first two years after the end of therapy. We can therefore conclude that the 6 months' regimen tested is not followed by relapses within the first two years after the end of therapy with a confidence limit of 11·9%.

Acknowledgments

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The future of leprosy in the Dominican Republic: experience with multidrug therapy

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Leprosy in the Dominican Republic

The history of leprosy in our country begins in the colonial days, even though many authors have tried to demonstrate that this illness existed among the natives. These theories have been refuted, as for instance, by Charlevoix in his book 'History of the Island', where he refers to leprosy as an illness 'only found in the city'. In other words, his remark suggests that leprosy did not exist in the island generally before colonial development.

Checking the chronicles, we found that in 1581 the 'San Lazaro Hospital' existed, built in agreement with Indian Laws.

In 1844 with our national independence, laws were made so that all persons with leprosy had to be sent to a hospital and isolated. This was the first legal decree about the disease in our country.

In 1922 the 'National Colony for Leprosy' was founded, the patients were attended by the congregation of 'Sisters Mercedarias' and the lepers were kept there until 1981 when they were taken to an asylum in a new building, where old people and invalids now live. By the end of 1965 there were 776 lepers registered; 414 (53%) of them had 'malign forms' of leprosy and 362 (47%) 'benign forms'.

Dr Huberto Bogaert and four assistant doctors, after gaining experience from other countries, started work in the Dermatological Institute in February 1966 with an aggressive campaign against leprosy. He initiated the programme of leprosy control which continues in the Dominican Republic today.

During the period 1966–72 a study was set up of the leprosy problem in the Dominican Republic and the resources needed for a control programme were defined. In this period treatment was limited to the use of oral sulphones and clofazimine for those suspected of resistance to the former. In 1972 an agreement of coordination was signed between the Dermatological Institute and the State Secretary of Public Health, giving to the Institute the responsibility of programming, directing and executing the programme all over the National territory.

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The new programme of leprosy control in 1973–77 established ambulatory treatment based on oral or injected sulphones. Clofazimine was given for cases clinically sulphone-resistant and rifampicin was also used for some cases.

In the period 1978–82, the therapeutic regimens followed guidelines of the PAS/WHO and regional advisors. Some of their objectives were the reduction of endemic leprosy with the diminution of the rates of incidence and prevalence, constant vigilance of Mitsuda-negative cases, active search of new cases, education of the community, etc. Therapeutic changes were then introduced, e.g. the use of rifampicin, 600 mg daily, and clofazimine, 100 mg daily, with a weekly checking of all cases with positive bacteriology for 2 months, and then continued with oral/or injected sulphone.

The programme for 1983–87 has, as its principal purpose, the reduction of leprosy in our country by the diminution of incidence and prevalence rates with integrated treatment for known patients and active new case detection. This programme includes the treatment with multidrugs of all cases of leprosy, and the revison of our recording systems with the introduction of the OMSLEP System. We intend to emphasize health education, early diagnosis and early treatment of all cases with multidrugs.

Based on available data in our information systems the following results have been obtained in the last 20 years:

The rate of new MB cases, related to the total of the new cases, was higher in 1978 with 0·78, declining slowly to 0·37 in 1985.

The rate of new cases from dermatological consultation has remained between 0·67 for 1979 and 0·54 for 1985. But we noticed that the yield of cases for every 1000 dermatological consultations has declined from 6·91 in 1974 to 0·54 in 1985.

The rate of new cases discovered in domicilliary contacts increased from 0·17 in 1974 to 0·26 in 1985, but the yield of case-finding for every 1000 examined contacts has reduced from 16·70 in 1974 to 6·03 for 1985.

The rate of newly discovered cases has remained between 0·08 for 1981 and 0·05 for 1985; but the yield for every 1000 patients has declined from 4·13 cases in 1974 to 0·73 in 1985.

The rate of new cases discovered by population surveys has diminished from 0·23 (1975) when the provinces with highest prevalence were examined, to 0·01 (1983). The yield for every 1000 persons declined from 9·94 cases (1973) to 0·10 (1985).

The rate of new cases with physical deformity (of 2nd and 3rd grade) has diminished from 0·30 in 1978 to 0·06 in 1985.

The rate of bacteriologically positive cases increasd from 0·18 in 1973 to 0·27 in 1985. This was due, at least in part, to improvement in our methodology and techniques in the taking of skin smears.

The rate of treatment non-compliance to treatment increased from 0·01 (1966) to 0·05 (1985), the pigmentation caused by clofazimine being the principal cause of treatment rejection.

The rate of leprosy reaction in multidrug cases diminished gradually from 0·31 in 1973 to 0·02 and has remained at that level since 1983.

The rate of the number of cases achieving bacterial negativity during the year, compared with those who started the year with positive bacteriology, increased from 0·08 in 1974 to 0·58 in 1985.

The rate of new cases in patients under 15 years was 0·13 in 1966. Subsequently there are two rises: in 1975 it went to 0·28 and in 1984 to 0·31. The incidence in this age group started with 2·03 for 1966, rose slowly to 5·64 in 1975, later dropping to 1·99 in 1985.

The incidence for all ages was higher in 1975 when it rose to 10·36, declining slowly until 4·38 in 1985. The prevalence rate also declined from 0·92 in 1980 to 0·67 in 1985.

Generally speaking there is evidence of earlier detection of new cases and an improvement in incidence and prevalence, with greater efficiency in our use of chemotherapy. With sustained effort we hope to achieve control and eventually eradication.

Experience in multidrug therapy

Since the Leprosy Control Programme started in 1966 in the Dominican Republic, until today, treatment has been changing from oral or injected monotherapy, or both, to non-supervised multidrugs for short periods, and finally to supervised multidrug therapy, starting in 1983, following the advice of the OMS Group of Experts. But we have made some modifications, mainly due to the fact that our programme is directed by a private, philanthropic institution. Our multibacillary cases receive supervised rifampicin 600 mg and clofazimine 300 mg, each month. Also self-administered clofazimine 300 mg and dapsone 600 mg each week. This treatment will be used for a minimum of 3 years.

The paucibacillary cases receive supervised rifampicin 600 mg and clofazimine 300 mg each month; and self-administrated dapsone 600 mg each week.

The monthly treatment is given by the country auxiliaries, in dermatology centres as well as in patients' homes, but the medical supervision is by the dermatoleprologist, who also carries out the patient re-examination every 6 months.

A review of our patients with MDT treatment shows the following results: From 4028 patients who received MDT in 12 regular doses or more, 9·2% were children of less than 15 years old and 90·8% were patients older than 15. Sixty-two per cent were paucibacillary forms of leprosy (Table 1).

Eighty-two per cent of the patients treated with MDT had previously received oral dapsone treatment, injectable dapsone, clofazimine and/or non-supervised rifampicin. The time of previous treatment ranged from 2 months to 15 years (Table 2).

Table 1. Number of cases in MDT, by age, sex and clinical group.

Clinical group	Age and sex					
	0–14 years		15 years and more		Total	
	Males	Females	Males	Females	No.	%
MB	31	16	976	517	1540	38
PB	171	154	891	1272	2488	62
Total	202	170	1867	1789	4028	100
%	5·0	4·2	46·4	44·4	100·0	—

Table 2. Number of cases with previous treatment or not, by clinical group.

Clinical group	Treatment					
	Previous		MDT only		Total	
	No.	%	No.	%	No.	%
MB	1211	78·6	329	21·4	1540	100·0
PB	2087	83·9	401	16·1	2488	100·0
Total	3298	100·0	730	100·0	4028	100·0
%	81·9	—	18·1	—	100·0	—

Table 3. Patients with treatment previous to MDT, with or without injuries.

Situation	Clinical group	With injuries			
		Clinical		Bacteriological	
		No.	%	No.	%
Cases with injuries cured with MDT	MB	353	38·5	121	78·1
	PB	462	50·4	—	—
	Subtotal	815	88·9	121	78·1
Did not cure		101	11·1	34	21·9
Total with injuries		916	100·0	155	100·0

There were 916 previously treated patients with clinical lesions when they started MDT. Three hundred and fifty-three MB cases (38·5%) and 462 PB cases (50·4%) were cured. In 101 cases (11·1%) the treatment was ineffective. In the patients with positive bacteriology, 121 cases (78·1%) became negative and 21·9% stayed positive (Table 3).

From the patients treated only with MDT, 656 had clinical lesions which disappeared in 109 MB cases (16·6%) and 323 PB cases (49·2%); in 224 cases

Table 4. Patients that received only MDT, with or without injuries.

Situation	Clinical group	With injuries			
		Clinical		Bacteriological	
		No.	%	No.	%
Cases with injuries cured with MDT	MB	109	16·6	74	53·2
	PB	323	49·2	—	—
	Subtotal	432	65·8	74	53·2
Did not cure		224	34·2	65	46·8
Total with injuries		656	100·0	139	100·0

Table 5. MB cases with treatment previous to MDT that inactivated clinically or bacteriologically, by number of months between the start of the MDT and the inactivation.

No. of months MDT	Clinical cure		Bacteriological cure	
	No.	%	No.	%
1–4	5	1·5	—	—
5–8	77	21·8	28	23·1
9–12	68	19·3	19	15·7
13–16	48	13·6	24	19·8
17–20	52	14·7	19	15·7
21–28	66	18·7	21	17·4
29–36	34	9·6	9	7·4
Ignored	3	0·8	1	0·8
Totals	353	100·0	121	100·0

(34·2%) lesions persisted. The bacilloscopy was negative in 74 MB cases (53·2%) and positive in 46·8% (Table 4).

Of the 353 MB cases with clinical lesions who had received treatment prior to the use of MDT, these lesions disappeared in 42·6% between 1 and 12 months of treatment with MDT. In 57·4% they disappeared after 13 to 36 months of MDT. In those who had positive bacteriology, 38·8% were negative in the first 12 months of treatment with MDT (Table 5).

Table 6. MB cases that inactivated clinically or bacteriologically receiving only MDT, by number of months between the start of the MDT and the inactivation.

No. of months MDT	Clinical cure		Bacteriological cure	
	No.	%	No.	%
1–4	—	0·0	5	6·7
5–8	33	30·2	15	20·3
9–12	30	27·5	15	20·3
13–16	15	13·8	11	14·9
17–20	16	14·7	11	14·9
21–28	11	10·1	10	13·5
29–36	4	3·7	7	9·4
Ignored	—	0·0	—	0·0
Totals	109	100·0	74	100·0

Table 7. Number of PB cases that kept clinically or histologically active to the 31.12.85 by months of MDT

No. of MDT months	Active		Total	
	Clinical	Histopathological	No.	%
1–4	—	—	—	—
5–8	—	—	—	—
9–12	20	12	32	18·1
13–16	19	21	40	22·6
17–20	21	22	43	24·3
21–28	27	20	47	26·6
29–36	9	6	15	8·5
Ignored	—	—	—	—
Totals	96	81	177	100·0
%	54·2	45·8	100·0	—

In the 109 MB cases with clinical lesions, treated with MDT only, the injuries disappeared in the first 12 months of treatment with MDT in 57·7% of the cases. In 42·3% of the cases, the lesions disappeared between 13 and 36 months of receiving the MDT. Of the 74 cases in which the bacteriology was negative, the change to negative occurred in the first 12 months of receiving MDT in 43·3% of the cases (Table 6).

Of the 2488 paucibacillary cases treated with MDT, 177 cases (7·1%) appear not to have been cured after receiving from 12 to 36 doses of MDT. Of the 177 cases, 96 (54·2%) are not clinically cured and 81 cases (45·8%) are still active on histopathology. We use histological control in our programme to verify healing in PB cases in which the clinical lesions have disappeared. The 18·1% of patients apparently not cured received 12 to 36 doses of MDT (Table 7).

Of the paucibacillary cases treated with MDT, 1050 were cured and subsequently discharged in 1985; 1261 remain under observation in 1986 without treatment (Table 8).

At this stage, we are disappointed by clinical and bacteriological results in some of our patients on multidrug therapy, who have failed to show the expected improvement. However, this may be related to individual variations in response to the drugs concerned. We are giving particular attention to the period of treatment required for a successful outcome in multibacillary cases.

Table 8. Number of PB cases without treatment after MDT, by situation, age or sex.

	Age and sex				
	0–14 years		15 years and more		
Situation	Males	Females	Males	Females	Total
Observation without treatment	128	123	431	579	1261
Healing discharge	42	45	380	583	1050
Total	170	168	811	1162	2311

SESSION II. TEST MODELS FOR THE EFFECTIVE CONTROL OF CHEMOTHERAPY FREE COMMUNICATION

Chairman A M DHOPLE (USA)

Lepr Rev (1986) **57,** Supplement 3, 137–148

The use of rodent models in assessing antimicrobial activity against *Mycobacterium leprae*

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Prior to the landmark discovery in 1960 of 'The Experimental disease that follows the injection of human leprosy bacilli into footpads of mice,'[1] the only means of searching for drugs active against human disease was to conduct clinical trials. Because clinical improvement of lepromatous patients is both very slow and variable, because the number of AFB (BI) in the skin falls extraordinarily slowly despite adequate therapy, and because the viability of solid-staining bacilli (MI) was not appreciated, early short-term clinical trials were difficult to conduct and the results even harder to interpret. Of the earlier studies on dapsone only the study of Lowe[2] followed a stable population until bacteriological negativity, finding 32 of 39 (83%) negative at 5 years, 31 of 35 (89%) negative at 6 years, and 34 of 35 (97%) smear-negative at 7 years.

The earliest studies on the effect of antimicrobial agents on *Mycobacterium leprae*-infected mice utilized primarily drugs known to be effective against *M. tuberculosis*. These first studies utilized constant treatment from the time of mouse footpad infection, generally with 5×10^3 *M. leprae*/footpad, either by incorporation of drug into mouse chow or daily (actually usually five times weekly) intraperitoneal injections. By these means Shepard[3] found dapsone, clofazimine, isoniazid, para-aminosalicylic acid, streptomycin, and cycloserine active and ethambutal and pyrizinamide inactive. By similar means Gaugas[4] in 1967 found the same drugs active, and notably, as well, rifampicin and cephaloridine. This method of drug testing, termed the 'continuous method', does not distinguish between purely bacteriostatic and bactericidal activity. Since it has been well established in bacterial endocarditis and a number of other infectious diseases where protective local or systemic host defence mechanisms are inadequate (osteomylitis, meningitis, and Gram negative bacteremia in the neutropenic patient) that bactericidal therapy is crucial to a salutary outcome and that the key to effective short-course chemotherapy for pulmonary tuberculosis is the inclusion of two or more bactericidal agents,[5] actual bactericidal activity against *M. leprae* is likely to be similarly important in the therapy of lepromatous

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leprosy. Since it was recognized, even before the importance of bactericidal therapy was determined to be important for most of these other infectious diseases, that purely bacteriostatic drugs were not likely candidates to be used in the therapy of leprosy, the 'continuous method' has been abandoned in more recent times.

Shepard,[6] recognizing the inherent problem with the 'continuous method', developed the 'kinetic method' to distinguish bactericidal drugs from those that are merely bacteriostatic. By this method mice are treated from day 60 to 150 after mouse footpad infection. Activity of a drug is measured in terms of the delay between 'plateau' in treated and control mice. A delay no longer than the period of time during which drug is administered represents bacteriostasis, whereas a longer delay which cannot be explained by drug accumulation represents bactericide. One pitfall in such studies is that drugs must be screened at maximal tolerated doses in order to determine whether a drug is truly inactive or merely bacteriostatic. Unfortunately, for many prospective agents such dosage information is not readily available. Utilizing the 'kinetic technique' Shepard[7] reported the following active drugs to be bactericidal: rifampicin, cephaloridine, dapsone, clofazimine, and ethionamide, and these others to lack such activity: isoniazid, gentamicin, cycloserine, PAS, clindamycin, streptomycin, and thiambutosine.

Colston[8] developed a procedure termed the 'proportional bactericide technique' which utilizes the mouse footpad analogously to an *in vitro* tube dilution and provides incontrovertible evidence of bactericide. In this procedure groups of mice are inoculated in both feet with 10, 100, 1000, and 10,000 *M. leprae* and treated for the initial 60 days; single feet are harvested and *M. leprae* enumerated 1 year after the conclusion of therapy, a time sufficient for regrowth of *M. leprae* from one or more *M. leprae* surviving the therapy. The percent bactericide can then be quantified by a most probable number calculation or the Spearman–Kärber technique. The latter appears preferable because it allows for direct confidence limits to be applied to the results,[9] is considerably more precise,[10] and does not assume a Poisson distribution,[9] i.e. a random dispersion of *M. leprae*, which from the work of Shepard[11] appears not to be the case. By this technique Colston has established the following: percentage bactericide dapsone 72%, 78%; clofazimine 96%, 98%; rifampicin 99·99%, 100%; ethionamide 97%, 99%; thiacetazine 42%; thiocarline, thiambutosine 0%.

The mouse footpad can be utilized to establish the minimal effective dietary concentration for a drug and by analysis of the resultant plasma concentration the minimal inhibitory plasma concentrations.[6,12–14] Minimal inhibitory dietary concentration: dapsone 0·0001%, rifampicin 0·0003–0·01%. Minimal inhibitory plasma concentration: dapsone 3, 4 ng/ml, rifampicin 0·1–3·0 μg/ml

Some of our own work over the past few years has been involved in screening, primarily by the kinetic technique, new agents in mice for their activity against *M. leprae*. For promising agents we try to establish a minimal inhibitory dietary and plasma concentration and evaluate their bactericidal activity and efficacy when

combined with established agents. These efforts are based on the obvious problem in leprosy chemotherapy that the armamentarium of useful drugs is small, intolerance to the established agents is not uncommon, drug resistance to all those agents utilized is appreciated and on the rise, and no new drugs have been brought to the patient since rifampicin in 1970.

Minocycline is a commercially available oral tetracycline which has proven safe on chronic administration. Our studies demonstrate that amongst the tetracyclines minocycline is unique in being active against *M. leprae*. We have established that the minimal inhibitory concentration of minocycline for *M. leprae* is exceedingly low and considerably less than levels easily obtained in plasma and tissues of patients treated with customary doses. Furthermore, in our studies minocycline has proven to be consistently bactericidal for *M. leprae*.

Previously, others[4,7] have found tetracycline itself inactive against *M. leprae*. In our studies doxycycline (0·02% in mouse chow) also was inactive. In three separate studies we found minocycline bactericidal against *M. leprae*-infected mice: in two studies by the kinetic technique, 0·04% dietary minocycline resulted in prevention of *M. leprae* multiplication for 270 days and for at least 180 days (study still in progress) after therapy was discontinued, and, in another study by the proportional bactericide technique, 0·04% dietary minocycline was found to be 99% bactericidal. Of the drugs used to treat leprosy only rifampicin has proved more bactericidal. This impressive activity found for minocycline is most likely to be the result of its being at neutral pH the most lipid-soluble tetracycline derivative, allowing for its penetration of *M. leprae*'s largely lipid outer capsule and cell wall to its ribosomal site of action. We have established minocycline's minimal inhibitory dietary concentration for *M. leprae* in mice to be 0·01% and, by analysis of resultant mouse plasma by an agar disk diffusion method utilizing the minocycline sensitive *Bacillus cereus* strain ATCC 1178, established the mouse minimal inhibitory plasma level to be $\leqslant 0{\cdot}2$ μg/ml. Also, we have demonstrated increased activity against *M. leprae* with dietary concentrations from 0·01% to 0·04%, which yield plasma concentrations from 0·5 μg/ml to 0·9 μg/ml. It is noteworthy that in man following usual therapeutic doses 2–4 μg/ml plasma levels are attained, skin levels exceed plasma levels, and minocycline appreciably penetrates nerves. Furthermore, we found minocycline additive or synergistic with dapsone, rifampicin, and kanamycin against *M. leprae*.

Draper[15] in an editorial extolled the virtues of development of drugs for leprosy that work at a cell wall locus. Since bacterial cell walls contain moieties that do not occur in animal cells, interference with bacterial cell wall function or inhibition of cell wall synthesis offers an excellent locus for selective toxicity without harm to the host. The extraordinary worth of the beta lactam antibiotics (penicillins and cephalosporins) certainly attests to the value of such a strategy. Cycloserine, being a structural analogue of D-alanine, acts as a competitive antagonist of the enzymes which link D-alanine molecules in the bacterial cell wall. Shepard[16] previously found that 0·5% cycloserine in mouse chow by the

continuous method was only very weakly active, only delaying and partially suppressing multiplication of *M. leprae*. Because we[17] found reason to suspect cycloserine to be unstable in diet, we prepared diets every 2 weeks, changed mouse feeders 2 times weekly, and stored diets refrigerated. Though in these studies other hydroxamic acid derivatives were found inactive, in two separate experiments cycloserine 0·5% and 2% by the kinetic technique resulted in growth delay greater than a year and often for more than 2 years. The minimal inhibitory dietary concentration was established at between 0·1% and 0·5%, since lower levels in mouse chow, 0·1%, 0·025%, and 0·0025% were inactive. Studies are currently in progress with cycloserine at 0·5% and 2% by the proportional bactericide method in order to quantify actual killing of *M. leprae* with cycloserine alone and combined with dapsone.

Beta lactam antibiotics may also prove useful in leprosy and are known to act on cell wall synthesis and to be synergistic for certain Gram positive cocci with other agents working at a ribosomal level, particularly aminoglycoside antibiotics. As we discussed previously, Shepard[6] and Gaugas[4] found cephaloridine by the kinetic technique to have bactericidal activity against *M. leprae*. Unfortunately, it required injection and proved nephrotoxic, thus being removed from the US market. We screened a number of cephalosporins and cephamycins; all were inactive, except cephradine at 0·5% in mouse chow. *M. leprae* counts were $2{\cdot}62 \times 10^4$ at the completion of therapy but $1{\cdot}4 \times 10^6$ 60 days later, which suggests that only bacteriostatic behaviour was observed. Also clavulanic acid, a penicillinase inhibitor, together with amoxacillin (100 mg/kg), appeared active, however again merely bacteriostatic. Unfortunately, clavulanic acid is most unstable in solution and diet, but both tablets containing amoxacillin and clavulanic acid and an injectable including ticarcillin and clavulanic acid are currently marketed. The minimal activity we observed against *M. leprae* may be enhanced if larger doses are employed. Certain infections in mice, particularly klebsiella, require 800 mg/kg.[18] Both ticarcillin + clavulanic acid and amoxacillin + clavulanic acid are currently being studied in our laboratory both by the kinetic and the proportional bactericide technique. It is noteworthy that a number of mycobacteria have been found to contain a penicillinase. Prabhakaran *et al.* 'Beta lactamase: An induced enzyme in *Mycobacterium leprae*?' American Society of Microbiology Annual Meeting, March 23–28, 1986) has found penicillinase activity in *M. leprae*.

Previously, Pattyn *et al.*[19] found by the continuous method that the minimal effective dose of streptomycin was 50 mg/kg weekly. These studies found no profound difference whether the streptomycin was given once, twice, or three times weekly. Pattyn *et al.*[19] found (by the proportional bactericide technique) that streptomycin 100 mg/kg twice weekly resulted in 93 and 81% killing respectively if therapy was begun 2 and 22 days after footpad infection. We[20] previously reported that daily intraperitoneal kanamycin (100 mg/kg), streptomycin (150 mg/kg), and amikacin (100 mg/kg) resulted in impressive killing

(respectively 99·7%, 97%, and 96% bactericidal), while gentamicin (20 mg/kg) and tobramycin (20 mg/kg) were inactive. Because these very high doses and frequencies of administration might be toxic for man and are certainly impractical, we attempted to see whether reduced dosage or frequency of administration was effective. The results suggest that for streptomycin both reduced frequency of administration, at least down to once weekly, and reduced dosage, as low as 12·5 mg/kg/day, are associated with significant bactericidal activity. The synergism of rifampicin and streptomycin previously found for *M. kansasii* and *M. intracellulare* infections in mice[21] appears also to hold for *M. leprae*. It is noteworthy that once-monthly rifampicin plus streptomycin was more active than either drug alone and extraordinarily potent, 99·96% ± 0·02% bactericidal. Perhaps streptomycin could be applied to certain once-monthly rifampicin regimens.

Published studies utilizing the proportional bactericide technique to assess killing of *M. leprae* by dapsone and rifampicin are limited and confined to constant and high dietary concentrations against only two strains of *M. leprae*. Colston *et al.*[8] found dapsone 0·01% in mouse chow to be 78% bactericidal for one strain and 72% bactericidal for the other, while rifampicin 0·003% and 0·01% were found 99·99% and 100% bactericidal respectively against these two strains. The antimicrobial therapy of leprosy in man results in a range of bioavailability quite different from the relatively constant levels found in such mouse studies. We thus studied the killing potential of dapsone and rifampicin over a wide range of mouse dietary concentrations that result in the broad range of levels which may actually be experienced by leprosy patients in the course of therapy.

The strain studied herein had been extensively studied previously in this laboratory and was known to be consistently inhibited by 0·0001% dapsone and 0·00003% dapsone, but not by 0·00001% dapsone.[21] In the present study 0·00001% and 0·0001% dietary dapsone produced no measurable lethal consequences for the bacillus. Thus we observed a discordance between the minimal inhibitory concentration and minimal bactericidal concentration for this strain of *M. leprae*. The ability of higher dietary dapsone concentrations actually to kill *M. leprae* is modest and similar to that reported by others. Low-level sulfone therapy, including 1 mg dapsone/day, sulphetrone,[22] and DADDS[23] maintains plasma levels above the minimal inhibitory concentration but near those required for bactericidal activity for the strain herein studied. Such therapy has resulted in treatment failure. This further suggests the possible importance of bactericidal and not just bacteriostatic therapy in the successful therapy of lepromatous leprosy. Conversely, the significant killing of *M. leprae* by both 0·001% and 0·01% dapsone in mouse chow, which resulted in levels maintained in man by the usual 100 mg dapsone therapy, might also serve to explain why patients resistant to 0·0001% dapsone but not to higher dietary levels when treated with full dosage (100 mg) dapsone daily improve clinically and bacteriologically.[24]

The highest studied dietary concentration of rifampicin (0·01%) resulted in

considerable bactericidal activity, again in agreement with previous studies of Colston *et al.*[8] using the same methods. On the other hand, lower levels of dietary rifampicin showed a progressively diminished ability to kill *M. leprae*. While Colston[8] found 0·003% dietary rifampicin to be 99·99% bactericidal, we found rifampicin 0·005% to be 90% ± 6% bactericidal and rifampicin 0·003% to be only 50% ± 18% bactericidal (not sufficiently different from untreated controls). Such major differences between these two studies suggest that differing strains of *M. leprae* vary considerably in their susceptibility to the lethal effects of rifampicin. This is not surprising as previously Holmes[13] had demonstrated, amongst different strains of *M. leprae*, a range of minimum inhibitory dietary concentrations of rifampicin from 0·0003% to 0·003%. Furthermore, where Rees[14] found the minimal inhibitory dietary concentration of rifampicin for *M. leprae* to be 0·0025%, Shepard[6] required 0·01% for his strain. Thus *M. leprae* strains appear to exhibit a range of susceptibility both to the inhibitory and bactericidal activity of rifampicin. In this respect the efficacy of monthly 600 mg rifampicin as advocated by the WHO raises serious questions as to whether sufficient duration concentration of drug at the active site of drug action is maintained, especially for certain relatively insensitive strains of *M. leprae*, such as that studied in this report.

Previously Levy[26] reported 0·0001% clofazimine in mouse chow to be purely bacteriostatic. We found 0·0001% clofazimine to have significant lethal activity against *M. leprae* and higher levels to be progressively more bactericidal. Though the pharmacokinetics of clofazimine in mouse and man are highly complex[26] and extrapolating from mouse to man hazardous, perhaps lower-dose clofazimine therapy, especially for certain people troubled by clofazimine discoloration, might be considered.

Because dapsone works in the folate pathway and sequential blockage of this pathway has proved synergistic against a number of aerobic bacteria, exploiting such potential for *M. leprae* appears attractive. Previously Shepard[7] found that trimethoprim was not active against *M. leprae* alone nor did it potentiate the activity of dapsone. For the past 10 years we have evaluated a number of dihydrofolate reductase inhibitors.[27,28] Many were found inactive alone and unable to potentiate the activity of dapsone, while certain ones were definitely active and potentiated the activity of 0·0001% dapsone but combined with dapsone were fundamentally bacteriostatic. A rational approach to generating potentially useful dihydrofolate reductase inhibitors for use in leprosy has been spearheaded by Professor Seydel.[29] Seydel has been interested in a number of dihydrofolate reductase inhibitors, especially brodimoprim for its potential use in leprosy because of:

1 Its increased binding to the isolated dihydrofolate reductase of *M. lufu* and *M. leprae*. *M. leprae* is uniquely sensitive to dapsone MIC 1 ng/ml; the nearest mycobacterial sensitivity to dapsone is found in *M. lufu*, 40 ng/ml.

2 Brodimoprim's demonstrably profound synergism (more than with TMP) with dapsone against *M. lufu*.
3 Its potency in even low concentrations in combination with dapsone against highly dapsone-resistant *M. lufu*.
4 Its pharmacokinetic similarities to dapsone in man (half-life approximately 30 hours; TMP's, one hour).
5 Its safety (brodimoprim does not bind to mammalian dihydrofolate reductase and is safe in chronic toxicity studies).

We screened brodimoprim (0·03%) and TMP (0·1%, as previously studied by Shepard) alone and in combination with dapsone (0·0001%) against *M. leprae* infection of the mouse footpad by the kinetic technique. Both TMP and especially brodimoprim appeared active in combination with dapsone. Brodimoprim plus dapsone resulted in a growth delay of about 120 days. In a second experiment, both dapsone and brodimoprim and SE-SC-60, another dihydrofolate reductose inhibitor were active but only marginally superior to dapsone alone. It is noteworthy, however, that peak *M. leprae* counts in the mice treated with dapsone + 2 levels of SE-SC-60 never attained levels reached by controls or other treatment groups. Particularly because mouse staphlococcal infections though quite sensitive *in vitro* to sulfamethoxizole/TMP are not ameliorated by such therapy, these relative inactivities that we found in mice may be a function of certain peculiarities in the mouse folate pathway as opposed to an inherent lack of *M. leprae* sensitivity. Because the two studies utilizing brodimoprim yielded somewhat conflicting findings, brodimoprim and SE-SC-60 alone and together with dapsone are being assessed again, this time by the proportional bactericide technique. The ability of SE-SC-60 and brodimoprim to inhibit growth of a fully dapsone-resistant strain of *M. leprae* is also being assessed.

Newer quinolone antibiotics, unlike the prototype drug naladixic acid, have a very broad bacterial spectrum of activity and provide not just significant levels in the urine but effective systemic levels. Cultivable mycobacteria are uniformly sensitive to ciprofloxacin (personal communication, Seydel). In our laboratory by the kinetic technique, at 0·1% in mouse chow, ciprofloxacin appeared active but only bacteriostatic. Recent industry pharmacokinetic data suggests that such dietary administration, even at much higher concentrations, results in poor gastrointestinal absorption and peak plasma concentrations of < 1 μg/mg; we are thus reevaluating this agent following gavage at doses which ought to yield considerably higher levels.

An animal model analogous to human lepromatous leprosy, wherein bacterial numbers approach 10^9 or more and which is similarly immunosuppressed, might allow a model of bacterial persistence and the examination of effective chemotherapy to eradicate persisters. Because clinical trials in leprosy are expensive and require many years to allow proper interpretation of outcome, an animal model suitable for chemotherapeutic experiments would greatly

facilitate the development and screening of candidate regimens. The normal mouse permits the number of *M. leprae* to reach a ceiling of only 10^6 and is immunologically intact, therefore not providing such a suitable model. The thymectomized irradiated bone marrow reconstituted mouse described by Rees[30] permits the development of larger bacterial populations in an immunologically compromised animal, but attempts to reproduce such a model in the United States have not been successful, owing to early animal mortality. The neonatally thymectomized Lewis rat (NTLR), however, does survive well and permits the development of large populations of *M. leprae*. Fieldsteel *et al.*[31] utilized this rodent in a number of experiments for this express purpose. First he established the minimal effective dose of dapsone which prevented multiplication of 5×10^3 bacilli in rat footpads, which was 5×10^{-5} g%, this resulted in the establishment of a plasma minimal inhibitory concentrations for dapsone in rats of 4 ng/ml.[31] Next he conducted a number of experiments so as to evaluate the killing potential of 5×10^{-5} g% dapsone in mouse chow and at 100-fold greater concentrations. Five $\times 10^{-5}$ g% dapsone resulted in no killing of bacilli, while 5×10^{-3} g% resulted in killing of bacteria at a rate similar to that found following dapsone therapy in man. However, neither dapsone alone (up to 0·005%) nor single doses of rifampicin (1 mg/kg, 5 mg/kg, 10 mg/kg, or 20 mg/kg) eliminated persisters.[32,33] Similarly, the combination of a single dose of rifampicin, 10 mg/kg, on a background of 5×10^{-5} g% dapsone in rat chow was not fully effective either. Finally, single doses of rifampicin (10 mg/kg) together with continuous dapsone, 5×10^{-3} g%, and continuous dapsone 5×10^{-5} g% together with up to 10 doses of rifampicin resulted in viable bacilli still being present and detectable upon mouse of NTLR subpassage.[33] Thus a model of persisting *M. leprae* in an immunologically compromised rodent capable of permitting very high levels of bacilli has been created which cannot be sterilized by at least some reasonably potent multidrug therapy. The stage was hence set to try other and perhaps more efficacious therapy in an attempt to eliminate these persisting bacilli. Thus we decided to study more intensive regimens of dapsone and rifampicin in heavily infected NTLR.

Because of the large groups of animals required and the consequent expense, there have been only limited trials in experimental animals on the potential for synergistic antimicrobial therapy. However, Shepard[34] has tested in mice 30 combinations of two to three of the four clinically utilized bactericidal drugs (dapsone, rifampicin, clofazimine, and ethionamide). Twenty-three of these combinations showed a significantly increased killing of *M. leprae* over that of the most potent single agent of the combinations. However, only combinations containing clofazimine + rifampicin or dapsone + ethionamide in no instance demonstrated antagonism. Thus, these combinations are being studied in order to assess their potential for sterilizing established *M. leprae* infections in NTLR. Because rifampicin and ethionamide appear to be the most bactericidal agents established and in use in therapy of leprosy, this combination also will be

assessed. Thus in all, six therapeutic regimens in NTLR are being studied: 1, dapsone 0·005% + 10 doses of rifampicin 10 mg/kg by gavage; 2, dapsone 0·05% + rifampicin 0·01%; 3, rifampicin 0·01%; 4, clofazimine 0·01% + rifampicin 0·01%; 5, dapsone 0·005 + ethionamide 0·2%; 6, rifampicin 0·01% + ethionamide 0·2%.

In 1983 and 1984 four batches resulting in 100 NTLR (Charles River Labs) were inoculated in both hind footpads with 5×10^3 *M. leprae*. In order to provide an accurate baseline, at one year three NTLR from each batch were harvested and their hind footpads counted individually. All six control NTLR footpads from two batches that were harvested thus far one year following infection with *M. leprae* were shown to be uniformly infected with massive numbers of *M. leprae*, $> 5 \times 10^7$ per footpad.

Table 1. Number of *M. leprae* per footpad at 1 year.

NTLR	left foot	right foot
1	$7{\cdot}64 \times 10^7$	$1{\cdot}30 \times 10^8$
2	$3{\cdot}23 \times 10^8$	$5{\cdot}17 \times 10^8$
3	$7{\cdot}96 \times 10^7$	$5{\cdot}96 \times 10^7$
4	$6{\cdot}48 \times 10^7$	$5{\cdot}37 \times 10^7$
5	$6{\cdot}51 \times 10^7$	$5{\cdot}00 \times 10^7$
6	$2{\cdot}64 \times 10^8$	$7{\cdot}91 \times 10^8$

Because in recent years NTLR have not been found uniformly immunosuppressed and hence highly susceptible to massive multiplication and dissemination of *M. leprae*, these results are encouraging insofar as a uniformly highly infected NTLR model, simulating levels of infection of lepromatous leprosy, has been established in our laboratory. Beginning at one year groups of 15 rats were given four months of therapy as previously described: harvests on at least three of these treated NTLR are being performed at 2, 4, 6, 8 months and upon demise. Since it was decided to treat for 4 months, later harvests (6 and 8 months) from NTLR will enable determination of regrowth from any remaining 'persisters' after completion of therapy. The viability of *M. leprae* obtained from the footpads of all treated NTLR will be assessed in mice following inoculation of 5×10^3 bacilli per footpad and more sensitively assessed in NTLR by passage of 10^5 to 10^7 bacilli per footpad. If growth in passage rats is equivocal (< fourfold increase), *M. leprae* from these will be passed in turn as previously described to hind footpads of mice. Unfortunately, results are too fragmentary at this juncture to reach any conclusions. Immunotherapy of *M. leprae*-infected NTLR with interleukin 2,

gamma interferon etc., alone and combined with antimicrobials, is also being initiated in our laboratory.

The ability of various rodent systems to monitor chemotherapy trials in lepromatous leprosy and, in particular, to identify persisters is another issue of some interest. Though T/R mice were in part utilized to detect persisters in Malaysia following long-term dapsone[35] and rifampicin[36] therapy, it is not clear from those studies whether they proved more sensitive than normal mice in detecting persisters. Fieldsteel, in the normal mouse, utilizing larger inocula, 10^5 or 10^6 heat killed *M. leprae* with 10 live bacilli, by the ploy of subpassage was able to detect a smaller percentage of viable *M. leprae* than when utilizing a more customary inoculum size.

In two of our own publications[37,38] we demonstrated that the neonatally thymectomized Lewis rat provides a more sensitive monitoring system for detecting viable *M. leprae* in skin biopsies of lepromatous patients undergoing initial chemotherapy. This rat model allows for larger inocula (10^5–10^7) than the mouse as usually employed (5×10^3) but remains superior in detecting persisters to mice receiving even larger inocula (10^4–10^6). Furthermore, the neonatally thymectomized Lewis rat appeared superior in this respect, for unclear reasons, than the congenitally athymic or nude rat. Though not statistically significant in this small trial monitored by the NTLR, daily dapsone 100 mg plus a single dose of rifampicin 1500 mg appeared more effective than daily 100 mg dapsone plus weekly rifampicin 900 mg. A regimen, unlike the ones just reviewed, which could be found regularly to prevent multiplication of *M. leprae* from skin biopsies in the NTLR might be the most likely candidate for preventing persisters and allowing for safe discontinuation of therapy. On the other hand it may be, as is emerging from the WHO trials, that a minimum of 10–15% of the time even the most potent regimens leave persisters. Perhaps such patients are a subgroup of lepromatous patients, albeit a minority, with absolute anergy to *M. leprae*. Perhaps such patients will require lifelong antimicrobial therapy or in addition immunotherapy to effect a cure.

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Limited *in vitro* multiplication of *Mycobacterium leprae:* application to screening potential antileprosy compounds

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Inability to cultivate *Mycobacterium leprae in vitro* has been regarded as the bottleneck which obstructs research on modes of transmission, pathogenesis, immunization and the treatment of leprosy. The bottleneck in the cultivation problem always has been that of gaining significant information from an organism that has given no useful response to intensive inquiries by the disciplines of biochemistry, metabolism or biology. The two mycobacterial pathogens have been regarded as 'obligate intracellular parasites', because the efforts of several investigators have failed to induce their growth in bacteriologic media. The work on *M. leprae* has been continuous since shortly after its discovery by Hansen in 1873, a period of 112 years. *Mycobacterium lepraemurium*, agent of murine leprosy, was noncultivable since its discovery by Stefansky in 1903 until the convincing report of Nakamura in 1972, a total period of 69 years.

Table 1 offers a comparison of pertinent properties of *M. leprae* and *M. lepraemurium*. Both species fail to grow in pulmonary tissues of natural and experimental hosts (occasionally, *M. leprae* grows slowly in lungs of infected armadillos). This property is interpreted to mean that the oxygen sensitivity of *M. lepraemurium* will also be encountered in *M. leprae*. The table also reveals why *M. lepraemurium* was used earlier as an interium model for *M. leprae*.

Table 2 describes the significance of adenosine triphosphate (ATP) in such studies. In order to investigate obligate intracellular bacteria one needs a method that measures immediately and directly the physiologic status of unwashed cells. Bioluminescent determinations of ATP qualify for several reasons. ATP measures energy levels that are fundamental to biosynthesis and growth. During growth the increase in ATP per culture should coincide with microscopic counts.[1]

Figure 1 demonstrates the utility of ATP data while cells are growing and, even more importantly, while their metabolic systems are deteriorating. Curves A and M show that during growth of *M. lepraemurium* in Nakamura's medium the ATP per culture and microscopic counts are equivalent. Curve B, with 67% air

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Table 1. Properties of *M. lepraemurium* and *M. leprae*.

Property	*M. lepraemurium*	*M. leprae*
Intracellular habitat	+	+
Growth in pulmonary tissue	−	−
Ratios of single bacterial cells from tissue	4+	1+
Generation time in mice (days)	7	14–21
Dissemination from focal lesions (mice)	4+	1+
Fatal disease in mice	+	−
Growth in cell culture	+	−
Growth in cell-free system	+	+

Table 2. Significance of ATP.

1 ATP pools are extremely labile, either used rapidly for biosynthesis, exchanged with related nucleotides, or in damaged cells, degraded rapidly by ATPase.

2 Under constant conditions (e.g. *in vivo*), the ATP pools within bacterial cells are controlled by the net balance between rates of generating energy and rates of biosynthesis. ATP per aliquot or per culture measures functional biomass or cell numbers.

3 Minimal levels of ATP suffice for energy of maintenance; maximal levels promote maximum growth rates. Thus, ATP per bacterial cell can rank suspension of a given species in terms of growth potential.

space per culture tube, demonstrates the oxygen sensitivity of *M. lepraemurium* and reveals why oxidation-reduction potential must be regulated.[2] Curves C and D show the results when *M. lepraemurium* are incubated in typical mycobacterial media such as Dubos or Middlebrook 7H10. Note that ATP data define 25% air space as superior to 67% air space, even when the organism's metabolic systems are failing rapidly.

In our cultivation studies with *M. leprae* we have adopted two other parameters, besides ATP, to monitor the cultures, and these are uptake of tritiated thymidine (^{3}H-thymidine) by *M. leprae* and intracellular content of deoxyribonucleic acid (DNA).

Recent advances have resulted in an understanding of the process of DNA replication in prokaryotes. DNA is replicated by a multi-enzyme complex containing a relatively large number of different proteins. The replication is both semi-conservative and discontinuous.[3,4]

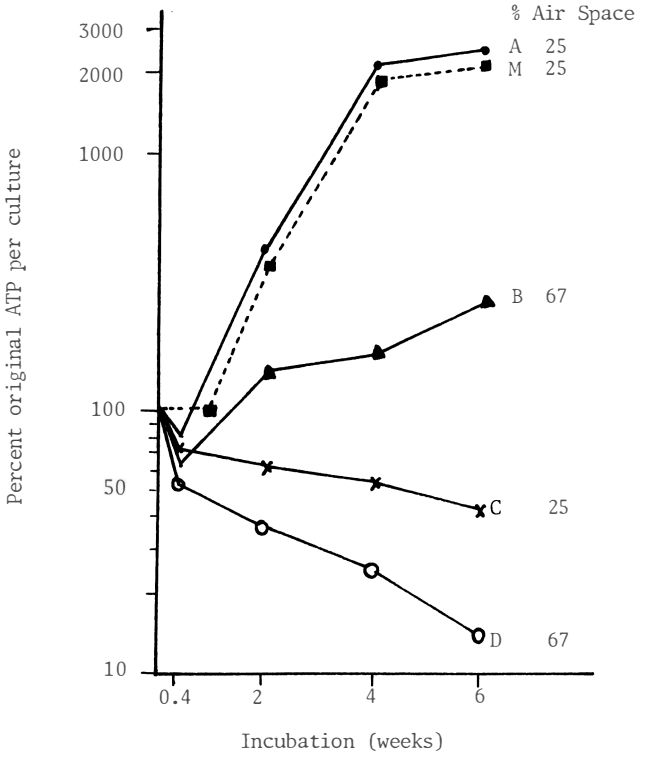

Figure 1. Significance of ATP determining in the cultivation studies of *M. leprae*. Data for *M. lepraemurium* incubated at 30°C.

Drutz & Cline[5] were the first to show that *M. leprae*, slowly metabolizing in human monocytes, are capable of incorporating ^{3}H-thymidine, presumably during synthesis of DNA, and provided an approach to evaluate at least the DNA-synthetic activity of this organism. Later Khanolkar *et al.*[6,7] found that *M. leprae* suspensions freed from mammalian cells would likewise incorporate ^{3}H-thymidine and also ^{3}H-DOPA. Subsequently, Nath *et al.*[8,9] showed that thymidine is incorporated by human-derived *M. leprae* in infected macrophages from mice and humans and used this technique to assess dapsone resistance of *M. leprae*. Since thymidine is a precursor of DNA but not RNA, its incorporation represents a fundamental correlate (DNA synthesis) with viability of *M. leprae*.

M. leprae suspensions were prepared by two different procedures, one by simply treating with trypsin[6] and the other by treating first with DNAase and then using Percoll density gradient.[10] These purified *M. leprae* were incubated at 34°C in presence of ^{3}H-thymidine. At various intervals samples were taken, washed either with saline and TCA or with TCA and methanol and counted in a scintillation counter. It was observed that uptake of ^{3}H-thymidine by *M. leprae*

was optimum after 24 hr. The best activity was obtained when the suspension was prepared by the Percoll gradient method and washed with TCA and methanol. With this the uptake of ^{3}H-thymidine by *M. leprae* was 0·12 pmol per 10 million *M. leprae* in 24 hr. It was interesting to note that under identical conditions uptake of ^{3}H-thymidine by *M. avium* and *M. tuberculosis* was very poor.

Incorporation of [^{3}H] thymidine into DNA: In order to use the [^{3}H] thymidine as an indicator of bacterial viability and DNA synthesis, it is important to determine if the [^{3}H] thymidine gets incorporated into bacterial DNA. For this purpose, three types of experiments were carried out: (a) direct assay of [^{3}H] thymidine into DNA isolated from pre-labelled *M. leprae*, (b) assay of thymidine kinase, and (c) assay of thymidine phosphorylase.

(a) Incorporation of [^{3}H] thymidine into *M. leprae*-DNA: *M. leprae* were labelled with [^{3}H] thymidine for 24 hr. In one set of experiments, uptake of [^{3}H] thymidine by *M. leprae* cells was measured. In second set of experiments, the labelled *M. leprae* (above) were used to isolate nucleic acids by the method of Wayne & Gross,[11] and the isolate was taken in Dimilume for scintillation counting. This gave the incorporation of [^{3}H] thymidine into total nucleic acids. In the third set of experiments, the pre-labelled *M. leprae* were further incubated for 24 hr in the presence of 0·3N KOH before extracting DNA and counting. This gave incorporation of [^{3}H] thymidine into DNA and the difference in the counts between the second and third set of experiments gave incorporation of [^{3}H] thymidine into RNA.

The results are presented in Table 3. Seventeen percent of the total [^{3}H] thymidine uptake by whole cells is attributed to the incorporation into DNA and only one percent into RNA. This is reasonably good evidence to justify using [^{3}H] thymidine uptake by *M. leprae* cells as an indicator of DNA synthesis.

(b) Assay of thymidine kinase in *M. leprae*: Thymidine, which generally has no role other than DNA building blocks, has the advantage of rapid conversion to the nucleotide level. Thymidine kinase (EC 2.7.1.75) is an enzyme of the pyrimidine salvage pathway which catalyzes the phosphorylation of thymidine to

Table 3. Incorporation of ^{3}H thymidine by whole *M. leprae* and *M. leprae* DNA.

	Incorporation of ^{3}H thymidine pmoles/10^{9} cells/24 hr	%
Whole *M. leprae* cells	12·70	100
Total nucleic acid	2·30	18
DNA (after KOH treatment)	2·22	17
RNA	0·10	1

thymidine monophosphate (TMP),[4] and with further phosphorylation, TMP is converted to TDP and then to TTP. The enzyme, DNA polymerase (EC 2.7.7.7) utilizes TTP to incorporate thymine (through precursor thymidine) into DNA. Thus, the incorporation of thymidine into bacterial DNA can be demonstrated by the presence of thymidine kinase (analyzing the reaction product, TMP). The modified method of Lee and Chang was adopted for this assay.[12]

There was a steady increase in DE-81 filter-retained CPM, which represent TMP. The thymidine kinase activity is linear up to 120 min. As seen in Table 4, during this period, the average value of thymidine kinase activity has been calculated to be 12·66 pmol/min/mg protein.

(c) Assay of thymidine phosphorylase in *M. leprae*: Thymidine phosphorylase (EC 2.4.2.4) within a few minutes converts thymidine to thymine and thus shuts off incorporation into thymidylate. This assay, in cell-free extract of *M. leprae* was performed by the method of Scocca, measuring spectrophotometrically free pyrimidine base (thymine) produced.[13] As seen in Table 4, thymidine phosphorylase is absent in *M. leprae*, another indirect piece of evidence which suggests that thymidine is converted to TMP and thus incorporated into *M. leprae*–DNA.

For comparison, assays for thymidine kinase and thymidine phosphorylase were performed with cell-free extracts of *M. lepraemurium* (*in vitro* grown), *M. lufu, M. avium* and *M. tuberculosis*. The results are presented in Table 4. It is interesting to note the relationship of [^{3}H] thymidine uptake by various mycobacterial cells to thymidine kinase and thymidine phosphorylase.

Another important development in the last decade is the successful transmission and growth of *M. leprae*. Yields of up to 10^{10} organisms per gram of liver have been found, thus supplying a constant source of organisms for such work. The armadillos source of *M. leprae* offers lessened metabolic variations in batch-to-batch preparations.

For all our *in vitro* cultivation studies, we used *M. leprae* suspensions purified by the Percoll gradient method. For ATP assays the aliquots of cultures were

Table 4. Thymidine kinase and thymidine phosphorylase in cell-free extracts of *M. leprae* and other mycobacteria.

Organism	Thymidine kinase*	Thymidine phosphorylase*
M. leprae	12·66	nil
M. lepraemurium (*in vitro* grown)	17·42	nil
M. lufu	9·37	4·21
M. avium	2·21	15·65
M. tuberculosis	0·85	22·37

* pmol/mg protein/min.

centrifuged, washed twice and treated with a mixture containing 0·1% each of trypsin, chymotrypsin and collagenase. Finally, cells were exposed to Triton X-100 followed by ATPase before extracting bacterial ATP. Triton X-100 selectively ruptures all the mammalian cells, liberating host ATP which is then destroyed by ATPase.[14]

Purified suspensions of *M. leprae* were inoculated in DH (Dhople), MY (Murohashi-Yoshida), Middlebrook 7H9 and Dubos broth (plus serum) media and incubated at 34°C for up to 20 days. At periodic intervals aliquots were taken and assayed for intracellular ATP. The results presented in Figure 2 suggest that *M. leprae* retain 75 and 54% of their original intracellular ATP in DH and MY medium respectively at the end of 20 days, while the loss of ATP is disastrous when incubated in 7H9 or Dubos medium. During the same period, the uptake of [^{3}H] thymidine by *M. leprae* was 86% and 72% of the original in DH and MY medium respectively. The data suggest that *M. leprae* remain metabolically active in both DH and MY media for at least 20 days. This is an important observation for cultivation studies and could not have been obtained by routine microscopic counts.

Table 5 shows the effect of incubating *M. leprae* in various media on its metabolic activity and viability.[15] As seen in Table 5, both DH and Mahadevan media support the maintenance of growth potential of *M. leprae* for at least 8 weeks. There is 20–30% drop over original in ATP levels and ^{3}H-thymidine uptake at the end of 4 weeks of incubation, which can be considered as the 'lag period'. This may reveal the serious decline in energy production even in the optimized system. However, between 4 and 8 weeks of incubation, the bacilli recover their metabolic integrity in these media. This represents the expansion of energy production and synthesizing useful membranes. The bacilli attain the original levels of ATP and ^{3}H-thymidine uptake by the end of 6 weeks. This can be interpreted as the stimulatory nature of these two media for maintaining the original growth potential of *M. leprae* and also the viability of *M. leprae* as evidenced by their ability to grow at the normal rates in the footpads of mice even from 4-week old cultures.

On the other hand, the effects of incubating *M. leprae* in either Murohashi–Yoshida, Dubos or Middlebrook 7H11 were disastrous. In Murohashi–Yoshida medium, by the end of 4 weeks ATP levels and capacity for ^{3}H-thymidine uptake had dropped to 50% of the original and declined steadily thereafter. The cells were removed at 4 weeks and later failed to multiply in the footpads of mice indicating that they had lost their viability also. The situation was still worse in the remaining two media.

Thus, the results suggest an excellent correlation between metabolic activity (ATP levels and capacity for ^{3}H-thymidine uptake) and viability of *M. leprae*.

There have been several reports of so-called successful *in vitro* growth of *M. leprae* in various media, but these findings could not be reproduced by other investigators in this field. We undertook studies to assess the fate of *M. leprae* in

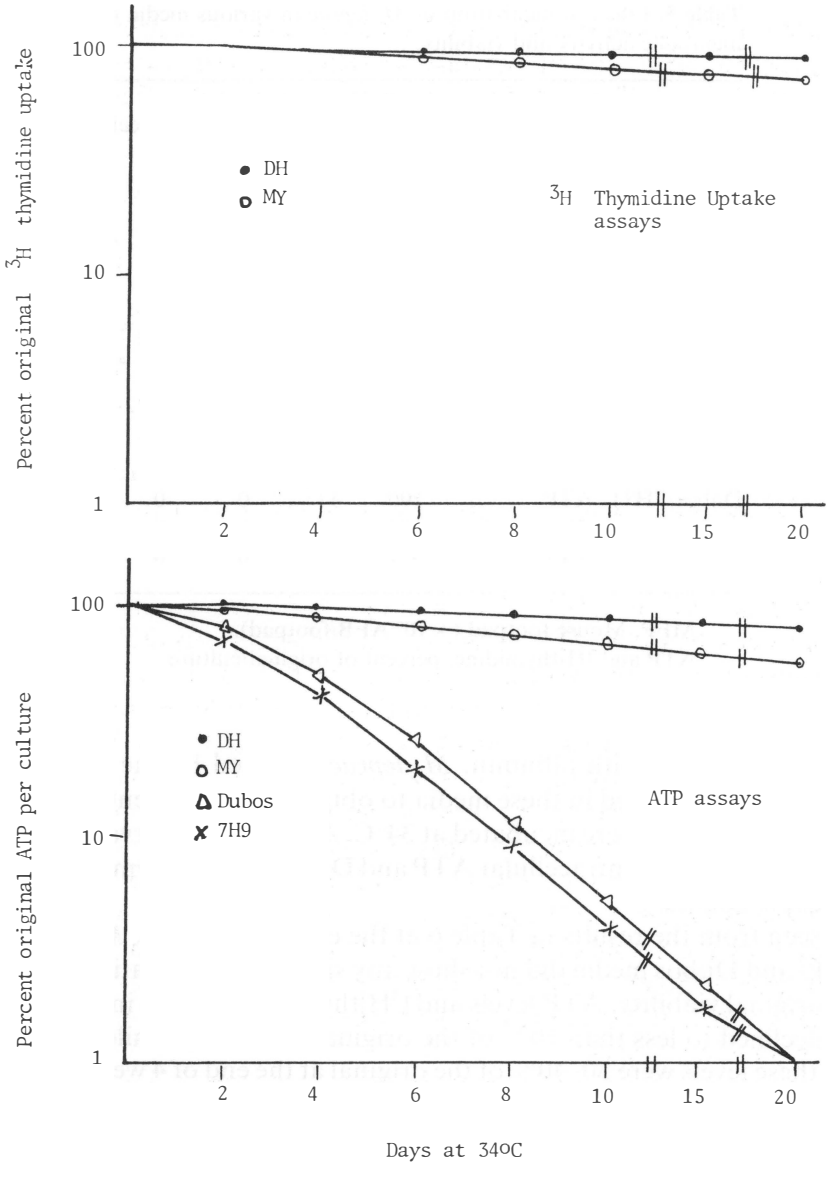

Figure 2. Stability of *M. leprae* in various media incubated at 34°C.

these six media; these included Dhople–Hanks medium (DH) that gave continuous *in vitro* growth of *M. lepraemurium*, Mahadevan's conditioned medium containing supernates from dorsal root ganglian cultures, Skinsness' hyaluronic acid medium, Murohashi–Yoshida medium (MY), Middlebrook 7H9

Table 5. Effect of incubation of *M. leprae* in various media on its metabolic activity and viability.

Medium	Activity	Incubation at 34°C for weeks				
		0	2	4	6	8
DH	ATP	100	80	72	95	138
	^{3}H-thymidine	100	82	70	102	149
	MFP	4·8		1·8	3·4	2·8
Mahadevan	ATP	100	83	77	102	149
	^{3}H-thymidine	100	86	78	105	132
	MFP	4·8		2·1	4·1	3·6
Murohashi–Yoshida	ATP	100	65	48	37	11
	^{3}H-thymidine	100	72	51	30	0
	MFP	4·8		0	0	0
Dubos/7H11	ATP	100	11	0	0	0
	^{3}H-thymidine	100	9	0	0	0
	MFP	4·8		0	0	0

MFP, Mouse footpad ($\times 10^6$ AFB/footpad).
ATP and ^{3}H-thymidine: percent of original/culture.

and Dubos medium with albumin. *M. leprae*, purified by the Percoll gradient method, were inoculated in these media to obtain initial concentration of 1×10^7 cells/ml, and cultures were incubated at 34°C. At periodic intervals, aliquots were taken for the assays of intracellular ATP and DNA, for [^{3}H]thymidine uptake and microscopic counts.

As seen from the results in Table 6 at the end of 16 weeks, *M. leprae* in MY, SK, 7H9 and Dubos media did not show any sign of multiplication and retention of any original viability. ATP levels and [^{3}H]thymidine uptake in 7H9 and Dubos media declined to less than 10% of the original 2–3 weeks while in MY and SK media, these levels were 30–40% of the original at the end of 4 weeks and less than 10% of the original at the end of 6 weeks. Cells recovered at the end of 16 weeks did not show any DOPA-oxidase activity and also failed to multiply in the footpads of mice.

On the other hand, both DH and Mahadevan media seem to be supporting the *in vitro* growth of *M. leprae*. At the end of 16 weeks of incubation, there was a significant increase in cell mass (400–600% of the original) as measured by all three criteria. The cells harvested failed to show any growth on any of the sterility test media, thus ruling out any possibility of these cells being other readily cultivable mycobacteria. These cells lost their acid-fastness when exposed to pyridine prior to staining, showed normal activity of oxidizing DOPA and gave standard growth curves in the footpads of mice 8 months after inoculation.

Table 6. *In vitro* growth of *M. leprae* in various media (pooled data)*.

Media	AD	MY	Mahadevan	Skinsness	7H9	Dubos
Counts	315–572	91–104	244–510	84–98	87–99	90–98
ATP	378–610	0	350–576	0	0	0
DNA	332–548	8–19	317–565	0	0	0
[^{3}H] Thymidine uptake	390–610	0	359–593	0	0	0
Pyridine extraction	+	±	+	±	±	±
DOPA	+	−ve	+	−ve	−ve	−ve
MFP†	1·36–5·83	−ve	0·73–3·42	−ve	−ve	−ve

* All values expressed as percent of original at 0 hour. All values for samples analysed 16 weeks after inoculations. Range of values from five different experiments.

† Mouse footpad harvests 8 months post infection ($\times 10^6$ *M. leprae*/footpad).

Data on Table 7 are representative of one of six experiments in the above series with DH and Mahadevan media wherein the results of three assays at periodic intervals are presented. There is a steady drop in the levels of bacterial ATP and DNA, and also a drop in ^{3}H-thymidine uptake for up to 4 weeks after which there followed a slow but steady increase in bacterial biomass, that reached optimum levels between 14 and 16 weeks. However, after 16 weeks there was no further increase in biomass, and the cells started deteriorating as shown from a rapid decline in the levels of all three growth parameters.

The cells harvested from DH and Mahadevan media from each of the six experiments above at the end of 16 weeks, were used to inoculate fresh DH and

Table 7. *In vitro* growth of *M. leprae* in DH and Mahadevan media. (Weekly findings of one representative experiment.)*

Weeks		0	2	4	6	8	10	12	14	16	18	20
DH medium:	ATP	100	80	72	95	138	242	368	475	494	398	117
	DNA	100	96	91	104	159	265	393	441	464	429	286
	MFP†									2·4		
Mahadevan medium:	ATP	100	83	77	102	149	265	378	454	468	331	74
	DNA	100	98	95	112	172	301	426	478	486	440	308
	MFP									1·1		

* All values expressed as percent of original at 0 hour.

† Footpad harvest 8 months post-infection ($\times 10^6$ *M. leprae*/footpad).

Table 8. *In vitro* cultivation of *M. leprae* in DH and Mahadevan media-transfer studies. (Weekly findings of a representative experiment.)*

Weeks		0	2	4	6	8	10	12
DH medium:	ATP	100	76	61	42	24	17	7·5
	DNA	100	95	90	81	69	54	43
Mahadevan medium:	ATP	100	71	55	38	19	8	4·8
	DNA	100	96	91	79	53	39	29

† All values expressed as percent of original at 0 hour.

Mahadevan media respectively. The inoculum sizes were the same as in primary cultures and all cultures were incubated at 34°C. The results pooled from five different experiments are presented in Table 8. During the 12-week period, ATP levels of *M. leprae* declined progressively to 3–10% of the original levels, while the capacity of *M. leprae* cells for ^{3}H-thymidine uptake was lost completely between the sixth and tenth week, thus suggesting that those cells were becoming more and more metabolically inactive throughout the incubation period. The cells harvested at the end of 12 weeks failed to oxidize DOPA as well as failing to multiply in the footpads of mice (Table 9).

Table 9. *In vitro* cultivation of *M. leprae* in DH and Mahadevan media-transfer studies.*

Media	DH	Mahadevan
Counts	92–107	93–106
ATP	3·2–9·4	3·8–6·2
DNA	31–58	22–41
[^{3}H] uptake Thymidine	0	0
†	−ve	−ve

Pooled data from five experiments. Inoculums were the cells removed at the end of 16 weeks from cultures of each of the five experiments of primary isolation (Table 6).

* All values expressed as percent of original at 0 hour and represent samples analysed at the end of 12 weeks.

† Footpads harvested 8 months post-infection.

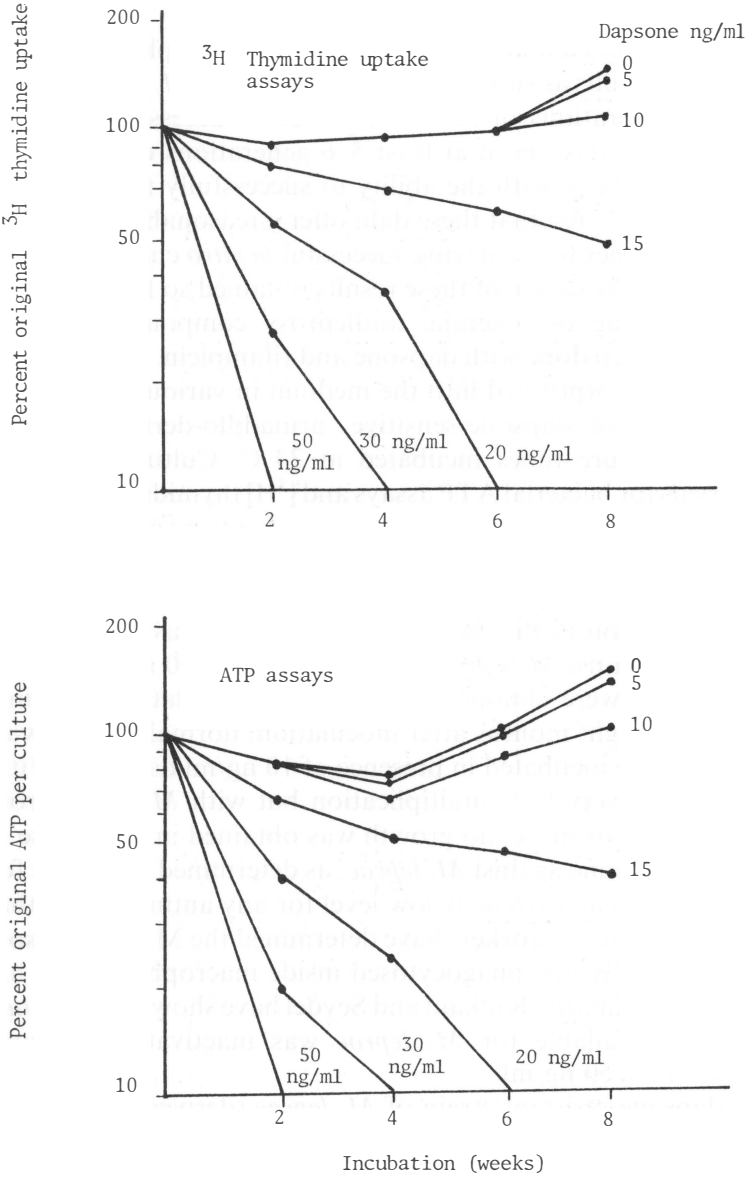

Figure 3. *In vitro* effect of dapsone on *M. leprae*.

Thus, it can be stated that there was a limited but definite multiplication of *M. leprae* in DH and Mahadevan media, but not in any of the other four media tested. Because of the techniques adopted, the declumping phenomenon can be ruled out. The question is whether the cells utilized nutrients and other substrates from the culture media or were able to multiply to a limited extent using their endogenous energy. However, the important point is that only DH and

Mahadevan media provided the necessary environments to the bacilli to survive for 4 months with limited multiplication. It should be emphasized that we are not considering these results as successful *in vitro* growth of *M. leprae* since there were less than three generations of cells in 16 weeks. Successful growth cannot be claimed unless there have been at least 5–6 generations of growth (30–60-fold increase in cell numbers) with the ability to successfully transfer these primary isolates. However, it is felt that these data offer a reasonable base upon which to conduct further studies for achieving successful *in vitro* cultivation of *M. leprae*.

We have taken advantage of these results obtained so far to develop a model for *in vitro* screening of potential antileprosy compounds and preliminary experiments have been done with dapsone and rifampicin. DH medium was used and dapsone was incorporated into the medium in various concentrations.

Known strains of dapsone-sensitive, armadillo-derived *M. leprae* were inoculated and culture tubes incubated at 34°C. Cultures were removed at periodic intervals for bacterial ATP assays and [^{3}H] thymidine uptake. The results are presented in Figure 3. No inhibitory effects were seen when the dapsone concentration was 10 ng/ml or less. At the end of 6 weeks, *M. leprae* became non-viable in presence of 20 ng/ml dapsone and this period decreased with increase in dapsone concentration in the medium. Thus, on the basis of these results, the MIC of dapsone against *M. leprae* is between 20 and 30 ng/ml. *M. leprae* from each set of cultures were obtained 8 weeks after inoculations and injected into footpads of mice. Eight months after inoculation, normal growth was obtained when *M. leprae* were incubated in presence of 10 ng/ml dapsone. With 15 ng/ml dapsone, there was very little multiplication but with *M. leprae* from cultures containing 20 ng/ml or more, no growth was obtained in the mouse footpads.

The MIC of dapsone against *M. leprae*, as determined by mouse footpad has been 3 ng/ml. This is an extremely low level for any antimicrobial that is in use today. Mahadevan and coworkers have determined the MIC of dapsone by an *in vitro* assay using *M. leprae* phagocytosed inside macrophages and have shown MIC of 28 ng/ml. Similarly, Kulkani and Seydel have shown that *M. lufu*, the best model organism available for *M. leprae* was inactivated by dapsone at a concentration of 30–50 ng/ml.

When a dapsone-resistant strain of *M. leprae* (derived from a nude mouse) was used in the above studies, dapsone at a concentration of 50 ng/ml did not have any inhibitory effects even after 12 weeks.

Using both dapsone-sensitive and dapsone-resistant strains of *M. leprae* in studies similar to the above, the MIC of rifampicin against *M. leprae* was found to be between 200 and 250 ng/ml (Figure 4).

So, that is where we are now and we plan to go forward from here, improving our medium to obtain continuous *in vitro* growth of *M. leprae*.

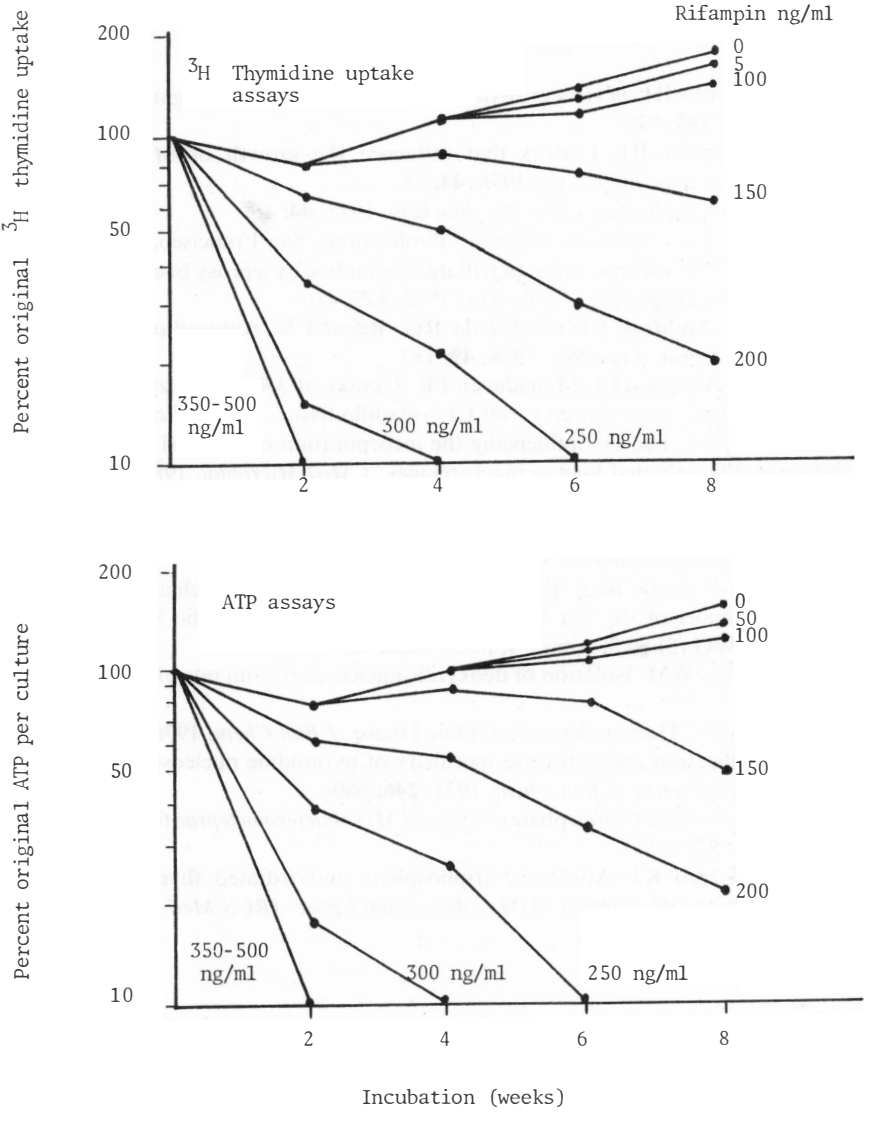

Figure 4. *In vitro* effect of rifampicin on *M. leprae*.

Acknowledgment

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Single bacterial cell mass analysis: a rapid test method in leprosy therapy control

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Introduction

A major characteristic of Hansen's disease distinguishing it from other bacterial infections is the fact that it is caused by a bacterial species not yet satisfactorily cultivable *in vitro*. Despite the severe problems which were and still are brought about by this fact, successful treatments of the disease, particularly the multidrug regime introduced by Freerksen & Rosenfeld[1] could be developed. Nevertheless, there remain several questions to be answered concerning: (i) the kinetics of the bacterial *in vivo* response to the chemotherapy—being associated with the dosage of the drugs; (ii) criteria for the duration of treatment; and (iii) the occurrence of drug resistance and persisters. For these reasons strong efforts have been made towards the development of techniques for the rapid detection of bacterial cell impairment and for *in vitro* drug screening.

Here, the groups of A M Dhople and P R Mahadevan should be mentioned in particular as they have developed *in vivo* systems for monitoring drug response of *Mycobacterium leprae* which are based either on an ATP-assay[2] or on EA rosetting of macrophages,[3] both applied to human biopsy material. Furthermore, both groups have extended their systems to *in vitro* drug screening by introducing artificial nutrients which not only keep host-derived *M. leprae* alive for several weeks but also allow for their limited multiplication.[4,5] We have described an *in vivo* test system based on the single bacterial cell mass analytical determination of the intracellular Na^+, K^+-ratio,[6,7] which we have just started to extend for *in vitro* drug screening.

This contribution will first give a short introduction to the general features of the technique, combined with a review of some already published results on human biopsy material and, secondly, the presentation of some very recent data from *in vivo* studies of drug kinetics and *in vitro* studies of drug action on armadillo-derived *M. leprae* in artificial media.

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Principles of the method and review

In Figure 1 the general procedure is outlined schematically. The bacteria are prepared on Formvar-filmed copper meshes (as used in electron microscopy) either from cell cultures or—in the case of the non-cultivable *M. leprae*—from biopsies via isolation from tissue, usually according to the protocol of Dhople[8] (see top). In each case the bacteria are washed quickly twice in distilled water to

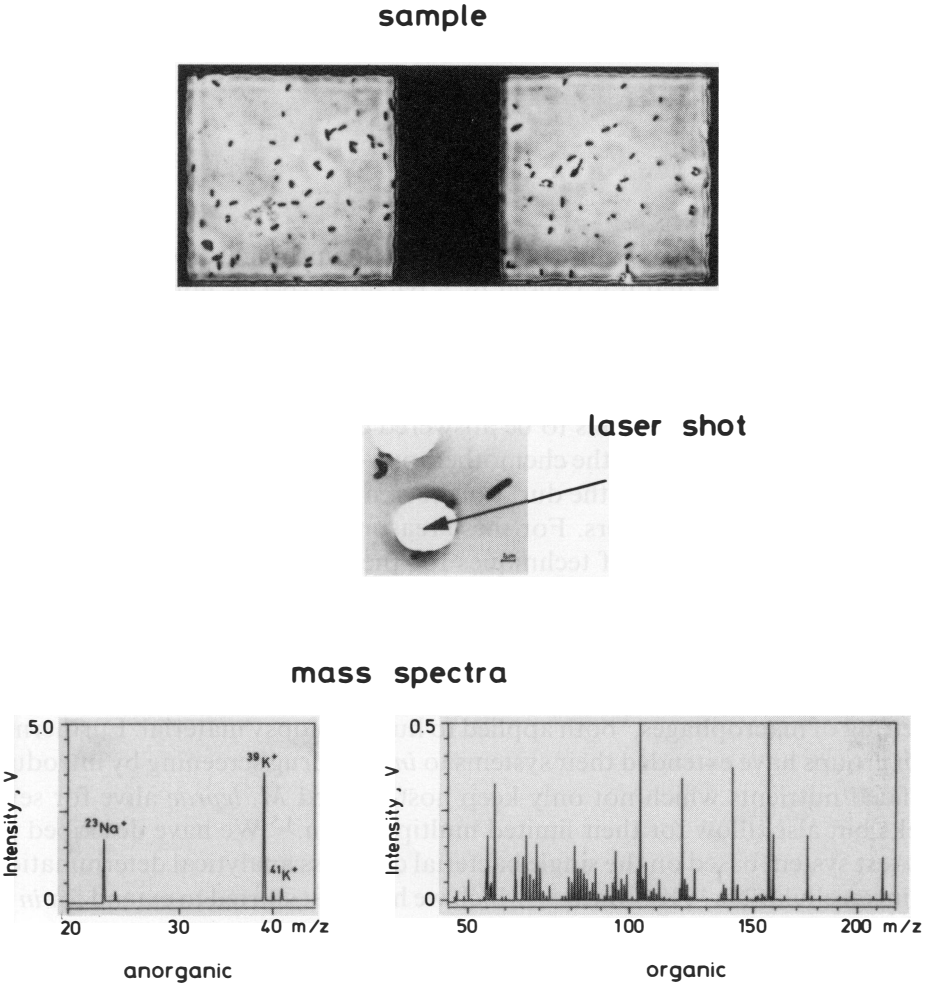

Figure 1. Schematic of single bacterial cell mass analytical procedure. Top: bacteria prepared on Formvar-filmed Cu-mesh (detail). Middle: laser-burnt hole in Formvar-film after vaporization of a bacterial cell (enlargement). Bottom: mass spectrometric information from a single cell on cation concentrations (left) or on organic fragment ions (right).

avoid contamination. The mesh is then mounted within a laser microprobe mass analyser LAMMA 500 (Leybold-Heraeus, Köln, West Germany).

A single cell at a time is vaporized, ionized, and subsequently mass analysed upon the irradiation with a high-power laser pulse (see Figure 1 middle). The produced so-called laser desorption mass spectra cover as well direct information on the intracellular cation concentrations of a single cell (see Figure 1 bottom, left) as indirect information on its organic matrix (see Figure 1 bottom, right). For a complete evaluation of a sample typically a few hundred cells are analysed this way, requiring only some hours. In first experiments with cultivable bacteria, mainly *E. coli*, we have shown that the ratio of the intracellular Na^+ and K^+ concentrations is a sensitive indicator of the physiological state of a cell and its alterations.

For this we have treated *E. coli* cultures with a chemotherapeutic and followed changes in the Na^+, K^+-ratio from 30 single cells prepared at different times after drug application. These results were compared with various other data like total cell number, $^{14}CO_2$-production, number of colony forming units, and ATP-release and were found to be in excellent agreement with these.[6] As an example, the single cell mass spectra of a viable untreated (left) and of a treated cell (right), here *M. leprae*, showing the drastic changes in the Na^+ and K^+ signals, are given in Figure 2.

Figure 3 shows, for the experiment described above, the comparison between single cell and the CFU-data.

A special feature of the single cell mass analytical technique is the possibility of establishing distributions of the Na^+, K^+-ratio within a larger number of

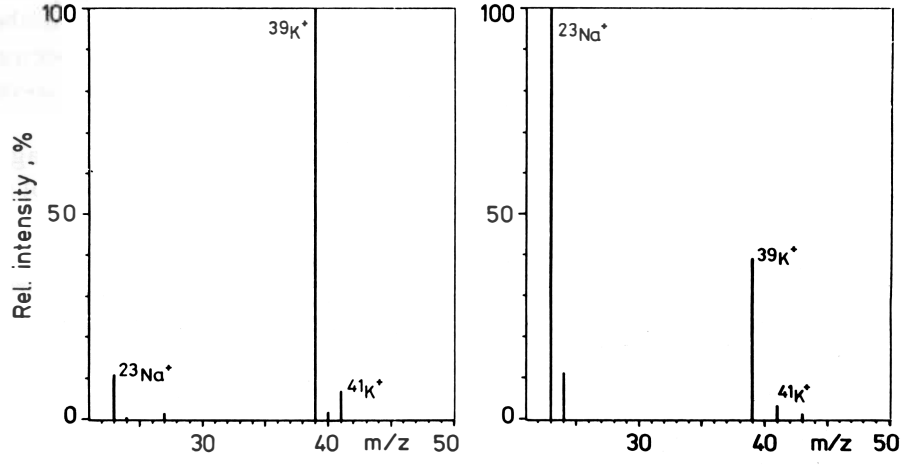

Figure 2. Cation signals from single *M. leprae* cells isolated from skin biopsies of a patient before (left) and 4 months after the beginning of DDS-monotherapy (right).

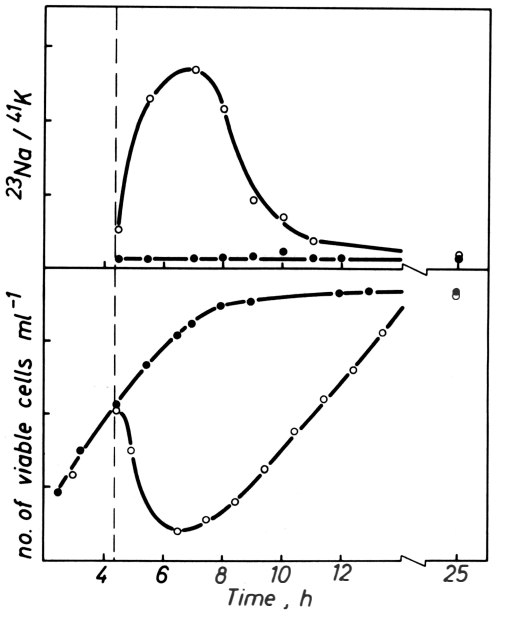

Figure 3. Time dependent responses of *E. coli* to treatment with the nitrofuran derivative HN32 at 5 fold the MIC. Top: $^{23}Na^+$,$^{41}K^+$-ratio averaged over 30 single cells from each measuring point. Bottom: viable cell number from plate counts. The drug was added at the time shown by the vertical dashed line.

single cells and this way having insight into the viability within a population. From this it should be possible to get well-founded information on the number of viable cells in isolated *M. leprae*. As mentioned above, the organic cell spectra can provide additional, but indirect information when they are treated as so-called mass fingerprints. Of course, the organic matrix of a bacterial cell is very complex and the interaction of the laser with the matrix leads to considerable fragmentation of the mostly very large biomolecules and thus to a larger number of small ions. These ions are registered as mass peaks in the spectra. Due to the unknown fragmentation pathways it is impossible—at least at the moment—to assign these mass peaks to particular molecules or functional groups. However, as mentioned already, the entity of all peaks can serve as mass fingerprints which are characteristic for a given bacterial sample. Applying sophisticated computer-aided mathematical multivariate cluster analytical procedures it is possible to obtain numerical relationships on the basis of which a separation between the various samples—either between different bacterial species or even between treated and untreated *M. leprae* cells isolated from human skin biopsies[7,9]—is possible.

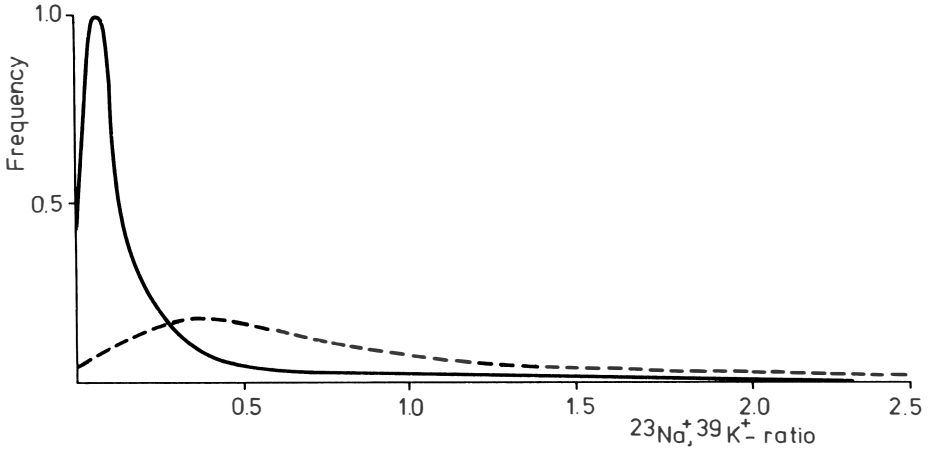

Figure 4. Distribution of the intracellular $^{23}Na^{+}$, $^{39}K^{+}$-ratios for two *M. leprae* populations of 400 cells each isolated from the same patient before (———), and 4 months after (– – –) the beginning of DDS therapy.

The applicability of the above outlined single cell technique to human-derived *M. leprae* could be demonstrated in an extensive study.[7] It could be shown that, in spite of the relatively tough isolation procedure, distributions of the intracellular Na^{+}, K^{+}-ratio within a population of 400 single cells isolated from the same patient before and 4 months after the beginning of DDS-monotherapy could be obtained, which resembles the findings from cultivable species (Figure 4). Furthermore, for about 40 patient biopsies—either untreated or under DDS-monotherapy for different periods of time—a good agreement between the results from intracellular Na^{+}, K^{+}-ratios and those from the ATP-assay (and, where performed, also those from mouse footpad test) could be stated.

Interestingly, from a combination of the cation measurements and the fingerprint evaluation it was suggested in two cases, that the bacteria were DDS-resistant. This was, in fact, later on confirmed by mouse footpad tests.

Actual results and discussion

The response to DDS-monotherapy as determined from changes in the intracellular Na^{+}, K^{+}-ratio of 88 human biopsy derived *M. leprae* samples after different periods of treatment—the preparations were kindly provided by A M Dhople—in dependence on the time of treatment, is demonstrated in Figure 5, showing a continuous increase until about 3–4 months after initiation of the therapy with no significant changes afterwards. This result represents the *in vivo* kinetics of the cell impairment due to drug action. From each sample—between

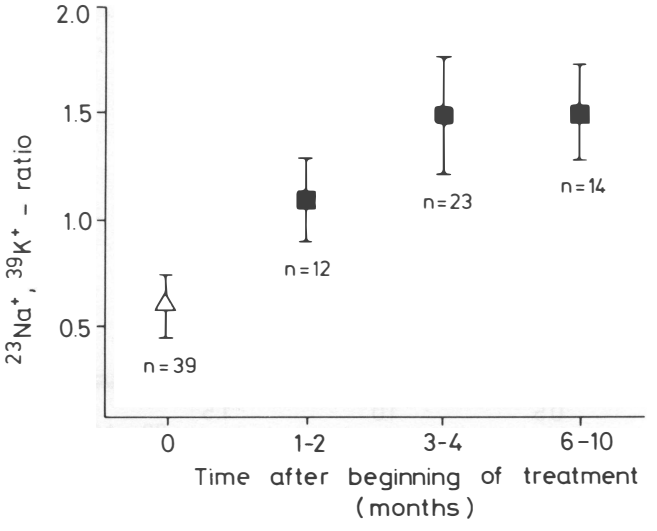

Figure 5. Time dependence of *in vivo* response of *M. leprae* to DDS therapy determined from the measurement of their intracellular $^{23}Na^+$, $^{39}K^+$-ratios after isolation from human skin biopsies. (*n* gives the number of patients included in each measuring point.) From each biopsy 100 single cells were analysed and the cation ratios averaged.

12 and 39 for the different periods of treatment—the average over 100 single cell values was taken. Even though this evaluation is very rough, especially according to the length of the measuring intervals, it may demonstrate the capacity of this technique for obtaining information on different drug combinations from *in vivo* studies. It may, furthermore, supply data for an optimal dosage (concentration and application intervals).

In a first series of experiments we have tested the applicability of the single cell method to *in vitro* drug screening. For this, aliquots of *M. leprae* isolated from armadillo liver were incubated in Middlebrook 7H9 growth medium at a concentration of approximately 10^9 ml^{-1}. To one batch DDS was added at a concentration of 5 μg ml^{-1}. After 24 hr the bacteria were prepared for cation determination.

Figure 6 gives the relative cumulative distributions of the Na^+, K^+-ratio within a population of 600 single cells in each case for bacteria analysed directly after the isolation from the liver (A), after inoculation in 7H9 medium (B), and after inoculation in medium + DDS (C). These results are compared with respective data from *M. smegmatis* also grown in 7H9 and harvested at the end of the exponential growth phase (D). From the relative cumulative distribution it can be deduced how many percent of the analysed bacteria have a Na^+, K^+-ratio smaller than a given value. Thus, for example, from the *M. smegmatis* sample about 90% of the analysed cells have a Na^+,K^+-ratio below 1, whereas from the

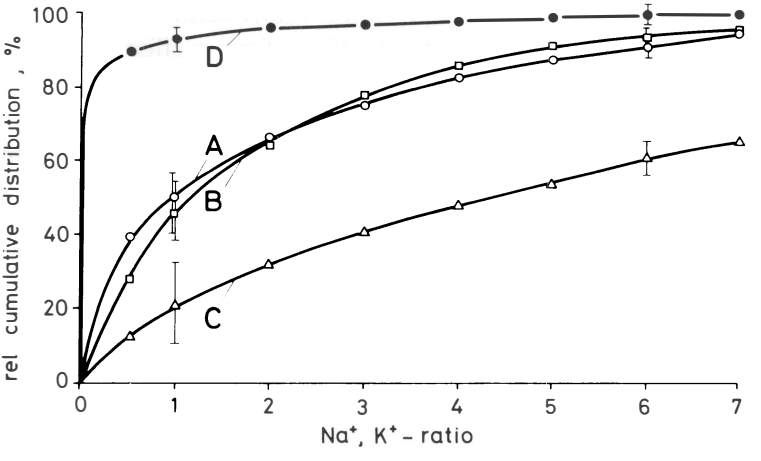

Figure 6. First results on *in vitro* drug screening showing the relative cumulative distributions of the $^{23}Na^+$, $^{39}K^+$-ratios within populations of 600 single cells in each case for: A, *M. leprae* isolated from armadillo liver; B, as A but after inoculation in Middlebrook medium 7H9 for 24 hr; C, as B but under action of 5 μg ml^{-1} DDS; and D, *M. smegmatis* grown in 7H9 and harvested at the end of exponential growth.

directly isolated *M. leprae* only about 45% have values below 1. For the DDS-treated *M. leprae* this figure decreases to about 20%.

Taking a Na^+, K^+-ratio of 0·6 as an average value for untreated, unimpaired *M. leprae* cells from skin biopsies (see also Figure 5) it may be concluded from Figure 6 that about 30% of the bacteria directly analysed after isolation from armadillo liver show this or an even lower value. This percentage (30%) is considerably higher than that of 0·1% given in the literature and based on transfer experiments to mouse footpads. Thus, it may be assumed, that the low percentage of multiplying cells in mouse footpads cannot be explained by a low number of *a priori* viable cells in the isolate but by adaptation problems to the new environment.

The discussion so far shows that the single cell mass analysis offers a possibility for differentiating between viable and impaired dead cells within a population. Curve C shows the influence of a drug (DDS, 5 μg ml^{-1}) on the Na^+, K^+-ratio of the armadillo derived *M. leprae* after 24 hr. Even when considering that the medium used (Middlebrook 7H9) does not support multiplication—but, as can be taken from curve C also does not affect the bacterial viability negatively within the observed interval of 24 hr—the drug action on the physiological state of the bacteria can clearly be derived. Of course, the drug concentration in these first measurements (each curve represents the average over three independent experiments) was comparatively high. However, we wanted to find out in this first step the general applicability of our technique for *in vitro* drug screening. This can

be answered positively, even though some improvements may be considered, concerning, in the first place, the introduction of more effective growth media as developed for instance by Dhople[4] and Mahadevan[5] and their coworkers. Nevertheless, it should be kept in mind that the aim in improving this method is the development of fast data acquisition, that means within a few days.

Acknowledgment

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Metabolism in *Mycobacterium leprae:* possible targets for drug action

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Introduction

Most of this paper deals with attempting to identify actual and possible targets for existing and future antileprosy agents. However, the application of some of this research into devising a suitable method for drug screening which uses [^{3}H]-hypoxanthine, i.e. radioisotopically labelled, will also be mentioned.

I want to emphasize biosynthetic pathways in *Mycobacterium leprae* because experience tells us that it is in these pathways that targets for antibacterial agents usually occur. This presents an immediate problem when considering leprosy bacilli since little work has been done on such pathways. Most of the work done on intermediary metabolism has been done on catabolic pathways, where a major finding is that *M. leprae* organisms can use a variety of carbon sources to release energy, by pathways including oxidative pathways like most other mycobacteria.[1]

A general scheme for many—but not all—biosynthetic pathways is shown in Figure 1. Examples of some pathways that fit the scheme in Figure 1 are shown in Table 1 and it is suggested that in such pathways, development of new agents should be directed against the synthesis of characteristic molecules from intermediates. This is because the intermediates in these pathways may be acquired directly from the environment by mycobacteria and in that case the synthesis of intermediates by mycobacteria themselves would be stopped. This is a useful thing for the bacteria to do since all biosynthetic pathways require a great deal of energy, and bacteria can save some energy by not synthesizing intermediates unnecessarily.

Although most mycobacteria can synthesize the intermediates shown in Table 1, when they have to, the detailed biochemistry of the pathways has rarely been worked out. Instead, the evidence for synthesis of intermediates is usually that mycobacteria—such as tubercle bacilli, *M. avium*—can grow in culture media

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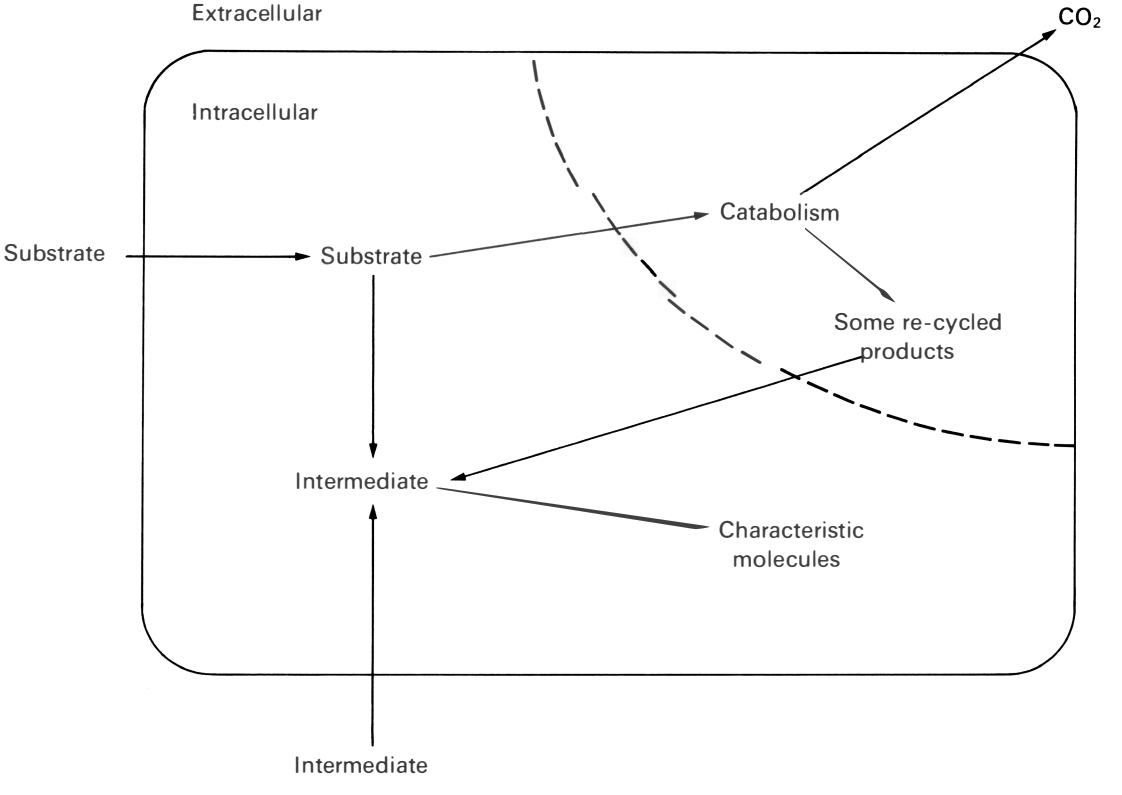

Figure 1. General scheme for biosynthesis of characteristic molecules. See Table 1 for examples of some important pathways. Only the area to the left of the broken line is discussed in this paper.

without a supply of intermediates. It therefore follows that in the case of *M. leprae*—which may have access to intermediates from the host—we do not yet know whether or not some intermediates can be synthesized at all.

There now follows a detailed discussion of four biochemical functions which involve characteristic molecules. Figure 1 illustrates the third and fourth examples but not the first two.

Biosynthesis of tetrahydrofolate

It is the first part of biosynthesis of tetrahydrofolate (Figure 2)—the production of dihydropteroate—that is only found in bacteria. Both the host and bacteria can convert folate to tetrahydrofolate. The well-known sulphonamide class of drugs act against the part of the pathway which is characteristically bacterial while benzylpyrimidines act selectively against bacterial, rather than mammalian

Table 1. Biosynthesis of some important characteristic molecules.

Extracellular substrate*	Intracellular substrate†	Intracellular intermediate†	Extracellular intermediate‡	Characteristic molecules synthesized
Simple carbon & nitrogen sources (e.g. carbohydrates, pyruvate, NH_3)	mainly tetroses pentoses and keto acids plus NH_3	amino acids	amino acids	Proteins
Simple carbon & nitrogen sources	pentoses glutamine aspartate C_1 units§	purine nucleotides	purine bases and nucleosides¶	Nucleic acids
Simple carbon & nitrogen sources	glutamine CO_2 aspartate pentoses C_1 units§	pyrimidine nucleotides	pyrimidine bases**	Nucleic acids
Carbon sources (e.g. acetate pyruvate glucose glycerol)	acetyl-CoA malonyl-CoA	fatty-acyl-CoAs	fatty acids	Complex lipids, e.g. mycolates, phenolic glycolipid

* Molecules obtained from the environment.

† Molecules which can participate in biosynthetic pathways (see Figure 1). Additionally *all* biosynthetic pathways require a great deal of energy which the bacteria must generate.

‡ Molecules obtained from the environment; all these examples have been shown to be scavenged by *M. leprae* organisms isolated from host tissue.[1]

§ Serine is an efficient source of C_1 units in the mycobacteria[19,20] and tetrahydrofolate is required for the use of C_1 units in these biosynthetic pathways.

¶ Purine nucleotides are not taken up directly by *M. leprae*[20,21].

** It is not known whether pyrimidine nucleosides or nucleotides can be taken up by *M. leprae*.

dihydrofolate reductase (see Figure 2). DDS[2] and a number of benzylpyrimidines (see contribution of J K Seydel in this supplement) have their primary sites of action in *M. leprae* against these two respective activities.

Uptake of iron

All living organisms require iron for their survival and growth. However, many bacteria growing in host tissue exist in an environment which is limiting for

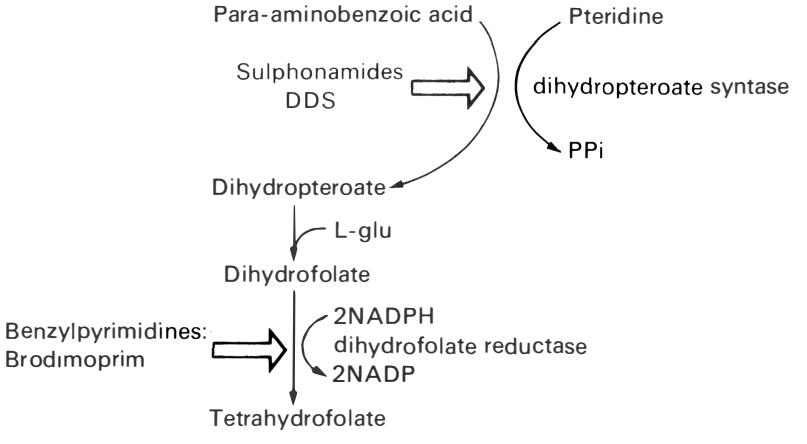

Figure 2. Latter stages of tetrahydrofolate synthesis.

available iron. Thus bacteria have evolved ways of scavenging iron using chelating molecules with a very high affinity for iron. Mycobacteria are no exception, producing iron chelators known as exochelins and mycobactins when grown in conditions with limited free iron.[3]

Exochelins are molecules which can chelate iron in the environment and present it to mycobacteria so that they take up the iron. It is not yet known whether *M. leprae* elaborates its own exochelins but when incubated with a panel of exochelins from nine different sources—from four strains of armadillo derived mycobacteria (ADMs: almost always isolated from armadillos infected with *M. leprae*[4]) *M. bovis* BCG, *M. vaccae*, *M. smegmatis*, *M. avium* and *M. neoaurum*—iron bound to the exochelins from one of the ADM strains and *M. neoaurum*[5] was taken up by *M. leprae*.

Since exochelins are used exclusively by mycobacteria for acquisition of iron, there appear to be some intriguing chemotherapeutic possibilities. Scandium and Indium can be chelated to bacterial siderophores and the resultant complexes were bacteriostatic to *E. coli*[6] and protected mice from infection with Klebsiella organisms.[7] Thus a preparation of exochelins might be used similarly to deliver toxic metals specifically to mycobacteria and as such might be of use in inhibiting growth of, or killing mycobacteria, including *M. leprae*.

Lipid biosynthesis

A most important metabolic activity in mycobacteria growing in simple culture media is the *de novo* fatty acid syntase, a multi-enzyme complex which has acetyl-CoA (C_2, i.e two carbons) as its initial substrate and adds further C_2 units, derived from malonyl-CoA, to it (see Table 1). The products are fatty acids, mainly C_{16} to

C_{32}.[8] However this complex is probably not a suitable target for potential antimycobacterial agents because its activity is repressed or inhibited in mycobacteria when fatty acids can be obtained from the environment. Such abolition of syntase activity has been shown in *M. convolutum* grown on alkenes[9] and *M. lepraemurium* grown on Ogawa medium, a lipid-rich medium.[10] Also, when cell-free extracts of *M. phlei* grown in the absence of lipids were assayed for *de novo* syntase with and without fatty acyl-CoAs, the syntase activity was strongly inhibited by fatty acyl-CoAs. I have recently shown that the uptake and incorporation into lipids of acetate into *M. microti* is strongly inhibited in *M. microti* grown in the presence of fatty acid or in mice (unpublished data) suggesting that in host tissue *M. microti* adapts to an environment where fatty acids are available for metabolic purposes.

Thus it may be necessary to search for targets in the biosynthesis pathways for characteristic, complex lipids for which fatty acyl-CoAs (derived from fatty acids such as C_{18} obtained from the environment) can be the starting substrates. Substrates and products of many pathways of lipid synthesis are likely to be restricted to mycobacteria, so any agents having a primary effect on these pathways would be very selective in acting against the bacteria, and not host enzymes. The likely biosynthetic pathways for α-mycolic acid (Figure 3) and phenolic glycolipid (Figure 4) are shown as examples of such pathways (see Minnikin (1982)[11] for review and further references). Many of the enzymes themselves have not been demonstrated. For instance, in α-mycolate biosynthesis (see Figure 3 for identification of letters) steps (C) and (D) are deduced from identification of intermediates in intact bacteria while (A) and (B) have been demonstrated in enzyme assays necessarily using cell-free extracts. Steps (E) and (F) are conjectural. Condensation reactions have been shown only using up to C_{10} acids in cell-free extracts of mycobacteria when two C_{10} acids condensed to give C_{20} acid.[12] But it would be so favourable in terms of energy conservation for the mycobacteria and in terms of the enzymes required to use awkward (long and very hydrophobic) substrates to carry out condensation reactions possibly using fatty acids scavenged from the host that it is likely that step (F) and probably even (E) are condensation reactions. However, it appears that *M. tuberculosis* can add C_2 units to fatty acids up to C_{54} acids if necessary;[13] so step (E) might be attained by elongation rather than condensation.

In the synthesis of phenolic glycolipid—or even characteristic molecules like phthiocerol, an intermediate (G) which has no counterpart in the host must be synthesized before an unusual addition of C_3 units (probably as propionyl-CoA: step H) occurs. Phthiocerol is probably converted to phenol phthiocerol dimycocerosate by step (M) shown with [^{14}C]-tyrosine followed by step (N). The incorporation of label from tyrosine suggests a role for step (L), although *p*-hydroxybenzoate is synthesized—only in bacteria—by the chorismic acid pathway (K). Step (N) requires fatty acids such as mycocerosate in *M. leprae* and tuberculostearate in *M. kansasii*, both synthesized (O) using enzymes which are

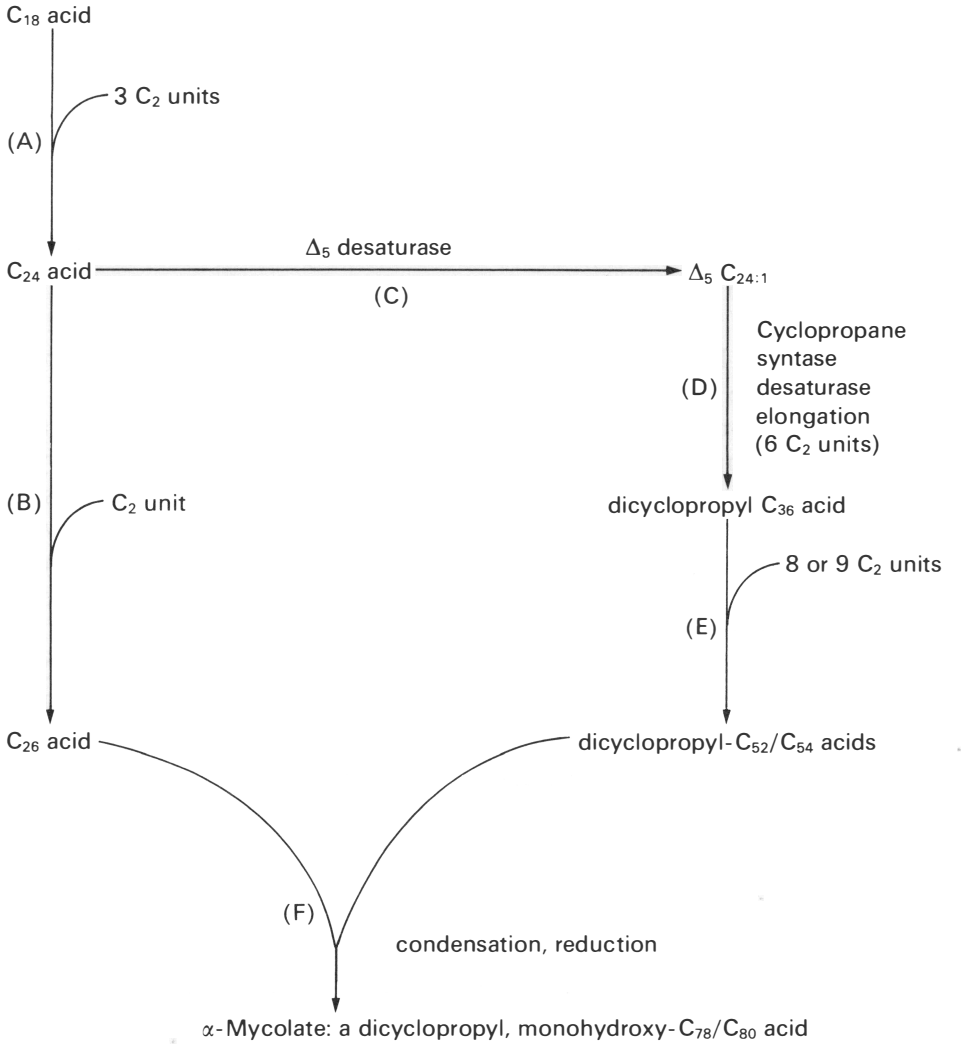

Figure 3. Synthesis of α-mycolate in *Mycobacterium tuberculosis*.

characteristically mycobacterial (for mycocerosate syntase[14]). Thus, in the synthesis of phenolic glycolipid from phthiocerol, there are four steps (K,M,N,O) which have both substrates and products synthesized only in bacteria—and some found only in mycobacteria. The final glycosylation step (P) also involves unusual sugars in many mycobacteria. Perhaps somewhere in these steps lies a target for a novel antimycobacterial agent!

What is probably needed now is a detailed study of the biosynthetic pathways of characteristic lipids of mycobacteria. The pathways seem promising for containing target enzymes for antimycobacterial agents because the substrates

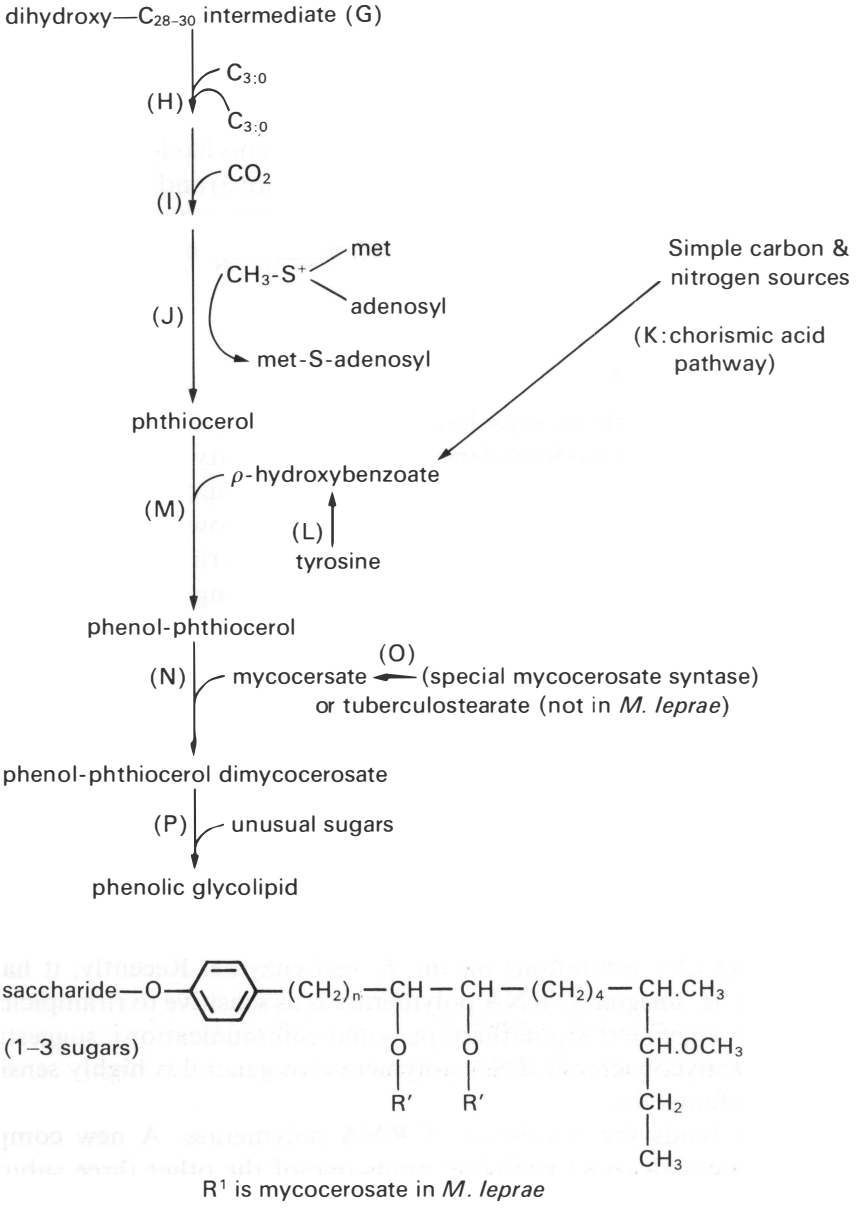

Figure 4. Synthesis of phenolic glycolipids.

and intermediates (Figures 3 and 4) are so different from any in mammalian systems, where fatty acids greater than C_{24} acids are not found. Thus agents acting directly on enzymes with characteristically mycobacterial substrates might be expected to have little effect on metabolism in the host. It would be most desirable to demonstrate individual enzymes, using cell-free extracts of mycobac-

teria. Only in this way, the effect of any agents could be tested on single enzymes. When agents inhibit an activity in whole bacteria (e.g. mycolate synthesis from, say, palmitic acid-C_{16}) it is always possible that they are having only a secondary, or indirect effect on the activity that is being measured. These suggestions may prove very difficult to follow up in *M. leprae*, but it seems likely that the inevitably complex biosynthetic pathways for α-mycolate (Figure 3) and phenolic glycolipid (Figure 4) will be conserved in whichever mycobacteria synthesize those complex lipids and thus, at least initially, easily grown model organisms could be used in place of *M. leprae*.

Nucleic acid biosynthesis

Nucleic acid biosynthesis in mycobacteria growing in relatively simple media such as Modified Dubos medium depends upon the ability first to use the carbon and nitrogen sources to synthesize nucleotides *de novo* and then the assembly of nucleotides into the nucleic acids, DNA and RNA. However, mycobacteria—including *M. leprae*[15]—can scavenge the purine and pyrimidine bases from the environment and convert them to nucleotides, thus saving a great deal of energy (see Table 1). Indeed, it is not known whether *M. leprae* can synthesize nucleotides *de novo* at all.

Thus, again, the best strategy seems to be to develop antimycobacterial drugs against the synthesis of characteristic molecules—the nucleic acids—from nucleotides. The substrates (nucleotides) for nucleic acid synthesis are common to both mammalian or bacterial systems so agents are needed which selectively inhibit the bacterial nucleic acid synthesis. Such agents are in use, and a notable example is rifampicin. Rifampicin is selectively active against its target—RNA polymerase—in mycobacteria amongst bacteria in general. Thus 10^{-8} rifampicin completely inhibits the *M. tuberculosis* enzyme[16] while having barely any detectable effect (2% inhibition) on the *E. coli* enzyme. Recently, it has been shown that the *M. smegmatis* RNA polymerase is as sensitive to rifampicin as the *M. tuberculosis* enzyme (Gopinathan, personal communication), suggesting the possibility that mycobacterial RNA polymerase in general is highly sensitive to inhibition by rifampicin.

Rifampicin binds the β subunit of RNA polymerase. A new compound, naphthyl-glycine hydrazide, probably binds one of the other three subunits. It inhibits the growth of *M. tuberculosis* and rifampicin resistant mutants are sensitive to it.[17] Because it acts on a different part of the same enzymes as rifampicin, there is the intriguing possibility that naphthyl-glycine hydrazide and rifampicin might act synergistically against mycobacteria.

Hypoxanthine incorporation for drug screening

During the work on purine metabolism in *M. leprae*, it became evident that the

Table 2. Incorporation of selected purines & pyrimidines into *M. leprae*.

Substrate	μM	Incorporation pmol/10^{10} *M. leprae*/24h
Hypoxanthine	1·2	8
	3·3	21
	17	74
	80	280
Adenosine	17	120
	80	465
Thymidine	0·07	0·06
	17	6
	80	19

Incorporation is into material insoluble in ice-cold 5% (w/v) trichloroacetic acid.

Substrate (in pmol) incorporated refers to pmol substrate supplied extracellularly and in calculary incorporation no account was taken of any possible dilution supplied substrate in intracellular pools of metabolites. For incubation conditions, see Khanolkar & Wheeler (1983).[15]

incorporation of radioactive hypoxanthine into acid insoluble material in *M. leprae* organisms[15] was far more rapid than radioactive thymidine incorporation (see Table 2). Adenosine incorporation was slightly more rapid than hypoxanthine, but also more variable. Thus the effect of a number of antileprosy agents on hypoxanthine incorporation was tested. Rifampicin and deoxyfructoserotonin inhibited the activity strongly.[15] Then the lowest concentration needed significantly (statistically: using Wilcoxon Rank-sum test) to inhibit hypoxanthine incorporation was determined for a number of other agents.[22] The concentrations were as follows: for DDS 10 ng/ml, for clofazimine 100 ng/ml, and for brodimoprim 30 μg/ml. These concentrations may be considered as MIC values for hypoxanthine incorporation in intact *M. leprae*. In DDS-resistant *M. leprae*, hypoxanthine incorporation was not inhibited by DDS (at up to 100 ng/ml) alone but with only 3 μg brodimoprim/ml (0·1 times MIC), almost complete inhibition of hypoxanthine incorporation was observed. A similar, synergistic effect of brodimoprim with DDS even in DDS-resistant mycobacteria is reported elsewhere in this book, and was shown previously.[18]

Since the variety of agents which inhibit hypoxanthine incorporation in *M. leprae* precludes them all having a primary effect on hypoxanthine incorporation,

it is likely that hypoxanthine incorporation is inhibited as an indirect effect—possibly, overall impaired metabolic competence of the intact *M. leprae* organisms incubated with the agents. If this is so then measuring hypoxanthine incorporation looks a very good way of screening novel, potentially antileprosy agents with a wide range of primary effects.

There seems little advantage in using substrates radiolabelled with extremely high specific activity in incubations with intact *M. leprae* since at the low concentrations of substrate which result (see Table 2) incorporation is almost proportional to the extracellular concentration of substrate, and adding unlabelled substrate or adding the same amount of radioactivity but using a substrate of relatively low specific activity has little effect on the amount of *radioactivity* incorporated.

Discussion and summary

I have emphasized the development of antileprosy drugs to inhibit synthetic pathways for characteristic bacterial molecules although at present the target enzyme dihydrofolate reductase has been shown to be a good target for possible new antileprosy agents.[18] However where intermediates are synthesized first (as shown in Figure 1 and Table 1) then characteristic molecules are made for the intermediates, it must be the second step which is sought to be inhibited. This is because, while intermediates (like nucleotides and fatty acyl-CoAs) need not be synthesized by mycobacteria all mycobacteria including *M. leprae* must synthesize their own characteristic molecules. Since products and often substrates in the synthesis of characteristic molecules are often only found in bacteria—or sometimes only in mycobacteria—such pathways ought to be able to be inhibited by agents acting highly selectively against mycobacteria. Finally, since many characteristic molecules of mycobacteria such as phthiocerol, phthiocerol based phenolic glycolipids, mycolates, and nucleic acids occur not only in *M. leprae* but also other mycobacteria, a search for new agents based on their synthesis might reveal agents which kill many pathogenic mycobacteria, not only *M. leprae*.

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Host-pathogen interaction— new *in vitro* drug test systems against *Mycobacterium leprae*— possibilities and limitations

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Introduction

The determination of the viability and drug sensitivity of *Mycobacterium leprae* in laboratory conditions has been a major problem, on account of the difficulty of growing this organism *in vitro*. To date the growth in the footpad of mice by the innoculated *M. leprae*, developed by Shepard[18] and later modified by Rees,[17] serves as the only recognized method to determine the viability of the organism, as well as evaluating the potency of antileprosy drugs. The major drawback of the mouse footpad test is that it is time-consuming and thus unsuitable for large numbers of tests or experiments involving several new compounds. However, several *in vitro* test systems to demonstrate viability and drug sensitivity of *M. leprae* have been described recently. They include the ^{3}H-thymidine incorporation method,[15,14] ATP quantitation method,[4] DOPA uptake system,[2] Fc receptor assay system.[3,6,12] Several other metabolic precursors have also been suggested as tools for determining viability of *M. leprae*. They are hypoxanthine, and amino acid;[8,9] labelled acetate.[19]

Recently Kvach & Veras[10] and Kvach *et al.*[11] have used a non-fluorescent fatty acid ester, Fluorescein-diacetate (FDA) and a nucleic acid stain, ethidium bromide (EB) for determining the viability of *M. leprae*. They showed that viable *M. leprae* convert FDA to fluorescent Fluorescein which accumulates within intact bacteria and make them fluoresce as green. The dead cells take up EB, due to defective membrane, and appear as orange red under the UV light.

In this presentation we would like to report several *in vitro* test systems that could identify viability of *M. leprae* and determine the drug sensitivity. The methods also enable screening new compounds for anti *M. leprae* activity. We would also like to show that these methods have excellent correlation with mouse footpad tests. In all these *in vitro* systems we have used *M. leprae* phagocytosed by macrophages and monitored various parameters to indicate viability or otherwise of the bacteria (Figure 1).

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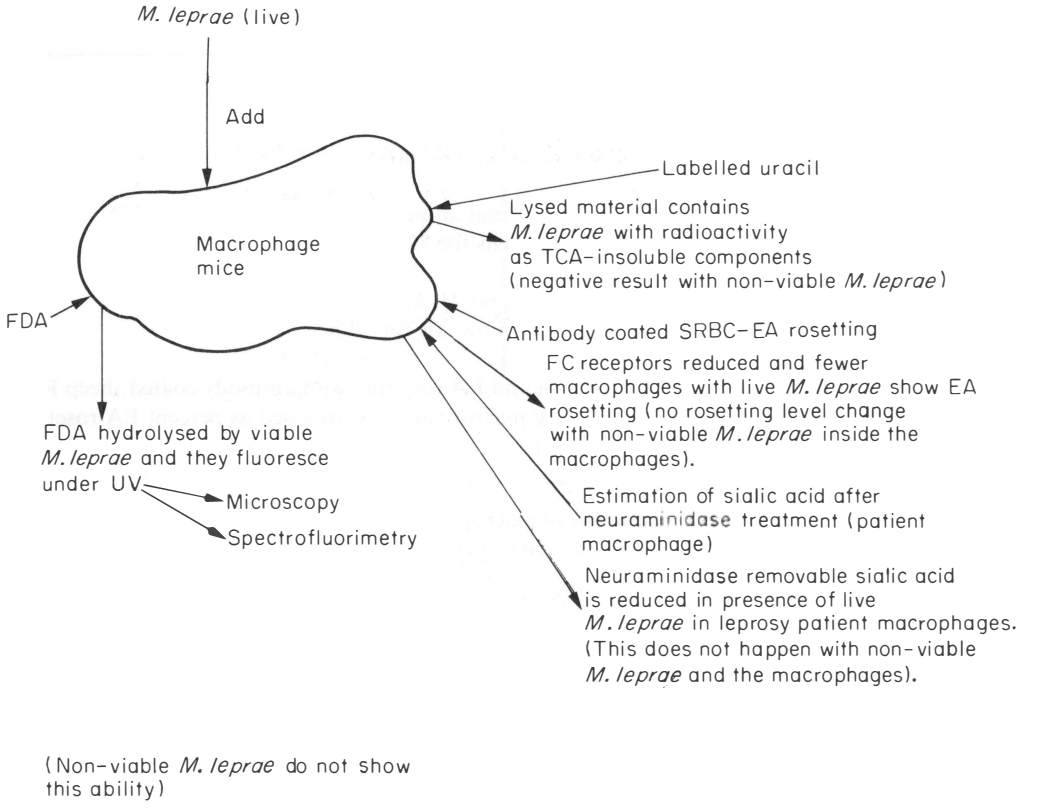

Figure 1.

Materials and methods

Mycobacterium leprae was obtained from infected armadillo tissues or human biopsies from untreated or partially treated lepromatous leprosy patients. Macrophages were from the peritoneal cavity of Swiss white mice. The preparations of both these components have been described in detail in our earlier publications.[6,12]

The Fc receptor assay system, using EA rosetting technique in relation to viability of *M. leprae* was described by Birdi *et al.*[3] using macrophages from humans. This was further adapted by using macrophages from peritoneal cavity of Swiss white mice.[12] The assay system is described in Table 1. Jagannathan and Mahadevan[6] had clearly demonstrated that dapsone, a drug used for *in vitro* assay systems, enters macrophages and the concentration inside the macrophages could be estimated in the solvent extracted sample by spectrofluorimetry.

Table 1. Fc receptor assay with macrophages *M. leprae* and drug.

Mouse macrophage (peritoneal) culture
↓
allowed to mature (3 days)
↓
DDS/RFP added and allowed to be exposed to cells for 72 hr
↓
Medium changed and *M. leprae* was added and exposed to cells for 72 hr
↓
Cells washed after removal of the supernatant and EA rosetting with antibody coated sheep RBC was done to determine Fc receptor expressing macrophages—(expressed as percent EA rosetting macrophages after scanning at least 200 cells).

Information background: The number of macrophages with Fc receptors is lowered by live *M. leprae* after their phagocytosis and it is unaltered by heat-killed *M. leprae* when compared to normal macrophages.[3]

Table 2. FDA assay with macrophages containing *M. leprae* with or without treatment with drug.

Mouse macrophages (peritoneal) (in 80% MEM + 20% human serum) allowed to mature 96 hr
↓
Add drugs
↓ 72 hr
Add *M. leprae* (armadillo/human 5×10^6/tube)
↓ 72 hr
Wash the cultures

- Used macrophages directly for FDA acid fast staining
- Lyse macrophages
  ↓ centrifuge
  separate the bacteria
  - FDA
  - Acid-fast staining
  - FDA
    ↓
    spectrofluorometry

FDA-EB assay system

This assay system was adopted using *M. leprae* phagocytosed by macrophages and was described earlier.[16] The basic steps in this assay system are given in Table 2. Further details on this method are under publication (Bhagaria and Mahadevan communicated).

Uracil uptake assay

The test system using the uptake of labelled uracil by *M. leprae* inside the macrophages has been described earlier (Vejare and Mahadevan, under publication). The broad details are presented in Table 3.

Table 3. Uracil incorporation test with *M. leprae* inside macrophages in presence or absence of drug.

Mouse peritoneal macrophages
↓
cultured for 3 days at 37°C (in 80% MEM + 20% human serum)
↓
infected with *M. leprae* (HK, live or RFP treated)
↓
24 hr later label with ^{3}H-uracil for 6 days
↓
After washing the cells, scrapped, count taken
↓
Lysed—freeze—thawed
↓
Centrifuged
↓
Bacteria counted and TCA insoluble radioactivity determined

Sialic acid assay system

The alteration in surface sialic acid due to viable bacteria inside the macrophages from patients has been reported.[1] The basic steps involved in the assay have already been published.[1]

EXPERIMENTAL RESULTS

Fc receptor assay system

That the *M. leprae* phagocytosed by macrophages are susceptible to drugs like dapsone and rifampicin is shown by the data presented in Tables 4 and 5 respectively. *M. leprae* in presence of drugs are unable to bring down the level of macrophages with Fc receptors but only live *M. leprae* are able to do so. The minimum inhibitory concentration effective in this assay system for dapsone is 0·028 μg/ml (28 ng/ml) and for rifampicin 0·11 μg/ml (114 ng/ml) (Tables 6 & 7).

Since the determination of EA rosetting is a subjective procedure, the technique of using ^{125}I labelled anti SRBC was adopted. The data presented in Tables 8 and 9 clearly show that this technique also shows the reduction in EA rosetting macrophages is quite clear in presence of live *M. leprae* and reversed in presence of anti-leprosy drug and *M. leprae*. In presence of live *M. leprae*, lesser amounts of labelled antibody bind to macrophages and this is reversed in presence of the drug with the *M. leprae*.

Lastly, using this assay system the viability of *M. leprae* exposed to dapsone and rifampicin in the macrophages, were determined in mouse footpad. It clearly showed that those *M. leprae* exposed to drugs and showing viability to lower the Fc receptors, also showed a poor growth in the mouse footpad. This indicated loss of viability by the *M. leprae* in presence of the drug inside the macrophages (Figures 2 and 3). The drug DDS showed that it acted clearly as bacteriostatic, since the *M. leprae* after removal from the drug-containing macrophage did show viability in mouse footpad. The rifampicin- (Figure 3) treated *M. leprae* showed loss of viability.

FDA-EB ASSAY SYSTEM

Data presented in Figure 4 show that in the presence of the anti-*M. leprae* drug dapsone the ability of *M. leprae* to break down FDA and express green fluorescence of the Fluorescein is drastically reduced. This could be due to the loss of viability in presence of the drug. A typical experimental result using this assay system with rifampicin is presented in Table 10.

Since determination of fluorescing *M. leprae* is a subjective experimental procedure, we have adopted estimation of fluorescence by viable *M. leprae* by

Table 4. Percent EA rosetting macrophages in presence of armadillo derived *M. leprae* with and without exposure to dapsone.

Expt. No.	Control (Møs only)	DDS only	Macrophages with: Live *M. leprae* only	DDS and *M. leprae*	Heat-killed *M. leprae* only
1	53	40	25	51	59
2	38	36	23	38	49
3	50	40	34	51	58
4	62	44	25	53	64
5	66	46	33	52	66
6	60	40	34	61	68
7	64	42	37	62	69
8	59	48	34	60	68
9	57	46	26	58	66
10	56	39	42	54	55
11	54	41	41	56	52
Mean ± SD	56 ± 2	42 ± 1	32 ± 2	54 ± 2	61 ± 2

Control, *M. leprae* added culture significant $p < 0{\cdot}05$; *M. leprae* only, drug + *M. leprae* significant $p < 0{\cdot}05$.

Table 5. Percent EA rosetting macrophages in presence of armadillo derived *M. leprae* with and without exposure to rifampicin

Expt. No.	Control (only Møs)	Rifampicin only	Macrophages with: Live *M. leprae* only	Live *M. leprae* and rifampicin	Heat-killed *M. leprae* only
	A	B	C	D	E
1	49	32	29	70	66
2	50	35	35	73	60
3	54	32	34	70	65
4	56	34	37	71	69
5	70	54	34	69	68
6	65	49	26	68	67
Mean ± SD	57 ± 4	39 ± 4	33 ± 2	70 ± 1	66 ± 2

P value A–C < 0·05 significant. *P* value D–C < 0·05 significant.

Table 6. Percent of macrophages exhibiting EA rosetting in presence of *M. leprae* and exposed to various concentrations of DDS.

DDS added per tube	Concentration of DDS (ng/ml medium)	Control macrophages only	Live *M. leprae* only	Macrophages containing: DDS only	DDS and live *M. leprae*	Heat-killed *M. leprae*
		A	B	C	D	E
10	14·2	49 ± 14	24 ± 6	30 ± 8	31 ± 9	55 ± 13
15	21·4	70 ± 7	46 ± 7	59 ± 3	59 ± 3	70 ± 0
*20	28·5	75 ± 5	48 ± 6	65 ± 5	76 ± 5	75 ± 5
50	71·4	72 ± 2	46 ± 8	60 ± 2	71 ± 2	72 ± 2
100	142·8	68 ± 3	42 ± 3	56 ± 9	70 ± 3	69 ± 5

Each value is an average of four experiments with each concentration of the drug added. A–B, $P < 0{\cdot}05$ significant; A–C, $P < 0{\cdot}05$ significant; A–D, $P < 0{\cdot}05$ significant up to 15 ng only; A–E, $P > 0{\cdot}05$ not significant; A–D, $P < 0{\cdot}05$ significant above 15 ng only.

* Minimum inhibitory concentration (MIC).

Table 7. Percentage of macrophages exhibiting EA rosetting in the presence of *M. leprae* and at various concentrations of rifampicin

REP conc. (ng) Per tube	(per ml) (ng)	Control Mθ only A	%EA rosetting (Mean ± S.D.) Mθ + *M. leprae* B	Mθ + RFP C	Mθ + RFP + *M. leprae* D	Mθ + *M. leprae* (H.K.) E
20	28·5	59 ± 3	31 ± 3	43 ± 7	49 ± 7	59 ± 3
50	71·4	65 ± 3	38 ± 4	51 ± 6	52 ± 5	66 ± 4
†80	114·2	65 ± 6	34 ± 3	51 ± 6	63 ± 4	64 ± 5
100	142·8	62 ± 4	36 ± 5	53 ± 4	62 ± 5	62 ± 1

* 3 experiments were carried out for each concentration and the mean ± SD is presented. 5×10^6/Leighton tube of *M. leprae* was used. Significance of difference (Student's *t* distribution test). *P* value is for data under each concentration of rifampicin used. A–B $P < 0{\cdot}05$; A–C $P < 0{\cdot}05$; A–D $P < 0{\cdot}05$ up to 50 ng only; A–E $P < 0{\cdot}05$; B–D $P > 0{\cdot}05$ up to 50 ng only.

† Minimum inhibitory concentration (MIC).

Table 8. Alteration in Fc receptor expressing macrophages in presence and absence of *M. leprae* as indicated by binding of ^{125}I labelled antibody coated SRBC.

Sample	Counts/400 s			
	1	2	3	AV ± SD
Control macrophages	772	438	423	544 ± 197
Macrophage + live *M. leprae*	188	142	142	157 ± 26
Macrophage + heat-killed *M. leprae*	692	535	310	512 ± 158
Macrophage + DDS (0·03 μg/ml)	266	230	190	228 ± 38
Macrophage + live *M. leprae* + DDS (0·03 μg/ml)	728	406	390	508 ± 149

The dose of *M. leprae*, 5×10^6/Leighton tube.

DDS dose—0·03 μg/ml—MIC level with Fc receptor system.

Table 9. Alteration in Fc receptor expressing macrophages in presence and absence of *M. leprae* as indicated by binding of ^{125}I labelled antibody coated SRBC.

Sample	Counts/400 S			
	1	2	3	AV ± SD
Control macrophage	595	540	434	523 ± 81
Macrophage + live *M. leprae*	214	274	158	215 ± 58
Macrophage + heat-killed *M. leprae*	518	613	410	534 ± 109
Macrophage + rifampicin (0·1 μg/ml)	372	378	236	328 ± 78
Macrophage + live *M. leprae* + rifampicin (0·1 μg/ml)	623	566	442	543 ± 92

The dose of *M. leprae*, 5×10^6/Leighton tube.

spectrofluorometry. Data presented in Table 11 show that with treatment of *M. leprae* inside the macrophages with rifampicin (1·14 μg/ml) the FDA degrading the fluorescing bacteria come down as indicated by reduction in the fluorescence level at an excitation wavelength of 485 nm and emission wavelength of 520 nm in the fluorimeter.

Lastly to show that the bacteria whose viability after rifampicin treatment was low, by FDA assay, were also tested in mouse footpad. The growth patterns of drug untreated *M. leprae* and drug treated *M. leprae* are presented in Figure 5.

Table 10. Effect of rifampicin on *M. leprae* inside the macrophages as indicated by their ability to break down FDA—an indication of viability.

	Phagocytosis (%)		No. of bacilli phagocytosed/ 100 macrophages		Viability (%)
	GFB	AFB	GFB	AFB	
Macrophages + live *M. leprae* only	70·5	92	423	2823	15
With 0·14 μg/ml rifampicin	4·5	90	18	2553	0·70
With 0·7 μg/ml rifampicin	3·2	85	8	2634	0·30

AFB, acid-fast bacteria; GFB, green fluorescing bacteria.

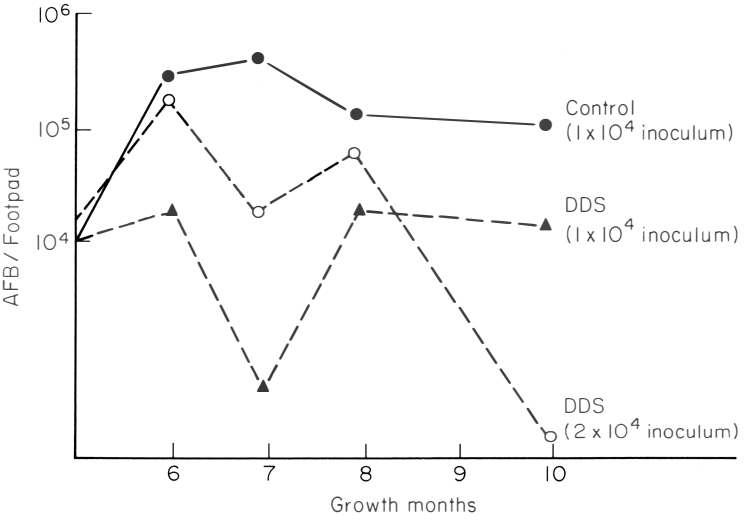

Figure 2. Loss of viability of *M. leprae* inside macrophages with dapsone (DDS) (*in vivo* assessment). Viability of Fc receptor: control, 80% rosetting macrophages; + *M. leprae*, 31% rosetting macrophages (loss of viability); + 0·03 μg DDS, 77% rosetting macrophages.

This clearly indicated that what appear as non-viable bacteria after RFP treatment by FDA test, also showed no viability in the mouse footpad.

URACIL UPTAKE SYSTEM

We had already demonstrated that uracil is taken up by free *M. leprae* and *M.*

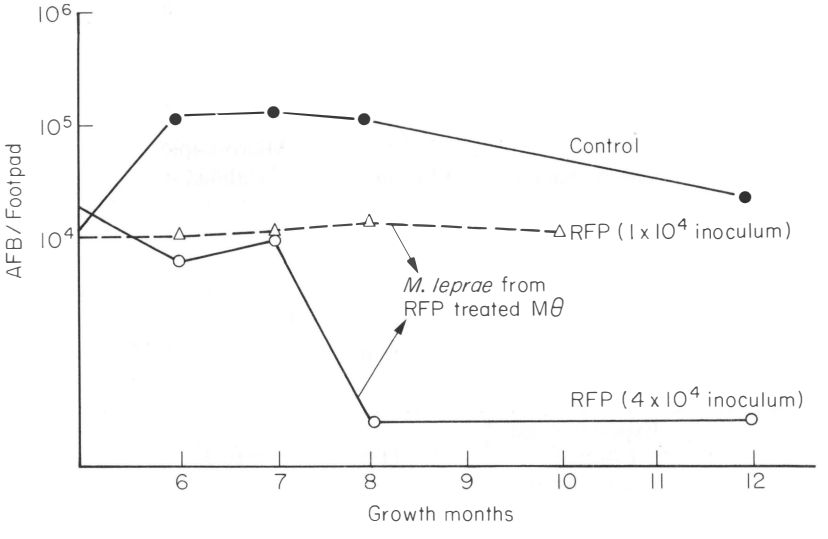

Figure 3. Loss of viability of *M. leprae* inside macrophages with rifampicin (*in vivo* assessment). Viability of Fc receptor: control, 50% rosetting macrophages; $+M.$ *leprae*, 31% rosetting macrophages; $+0{\cdot}1$ μg/ml RFP, 49% rosetting macrophages.

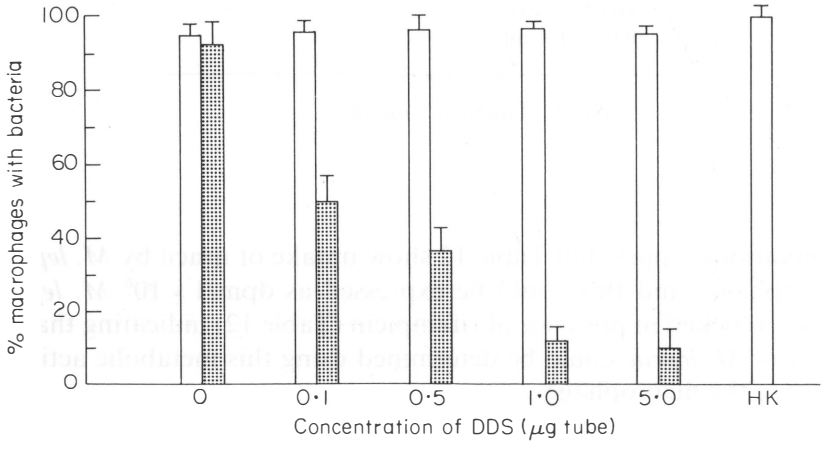

Figure 4. Effect of increasing levels of DDS on the percentage of macrophages with viable bacteria (green fluorescing) in relation to the total phagocytosis as indicated by the number of acid-fast bacteria (means $\pm$ SD of five experiments each with a count of 200 macrophages). Open columns, acid-fast bacteria; dotted columns, green fluorescing bacteria.

leprae inside the macrophages. We had also demonstrated that per unit amount of *M. leprae* more labelled uracil is taken up while the bacteria are inside the macrophages, as compared to the freely suspended bacteria (Vejare and Mahadevan, under publication).

Table 11. Loss of viability as indicated by reduced fluorescing bacteria determined by both microscopic count and spectrofluorimetry.

Sample/bacterial number	Fluorimetric reading	Microscopic viability (%)
Experiment no. 1		
Control $0{\cdot}3 \times 10^6$/ml	50·8	5·5
Drug treated $0{\cdot}3 \times 10^6$/ml	5·0	0
Experiment no. 2		
Control $0{\cdot}1 \times 10^6$/ml	11·8	10·3
Drug treated $0{\cdot}1 \times 10^6$/ml	2·5	0
Experiment no. 3		
Control $0{\cdot}05 \times 10^6$/ml	111·3	5·2
Drug treated $0{\cdot}05 \times 10^6$/ml	31·6	0

Drug, rifampicin 5 μg/ml.

Observations reported in Table 12 show uptake of uracil by *M. leprae* inside the macrophages and this could be expressed as dpm/1×10^6 *M. leprae*. This uptake was blocked in presence of rifampicin (Table 12) indicating that the drug sensitivity of *M. leprae* could be determined using this metabolic activity of *M. leprae* inside the macrophages.

SIALIC ACID ASSAY SYSTEM

We had reported that in the macrophages from bacillary negative lepromatous leprosy patients, live *M. leprae* induce a lowering of surface sialic acid.[1] This observation is also borne out by data presented in Table 13. This alteration of sialic acid level by *M. leprae* was shown to be both host and bacteria specific. Heat killed *M. leprae* were not able to do so. Thus, extrapolating this information we have also demonstrated that if viability of *M. leprae* is reduced by rifampicin (5 μg/ml) inside the macrophages, then they also lose the ability to reduce the surface sialic acid level of macrophages.

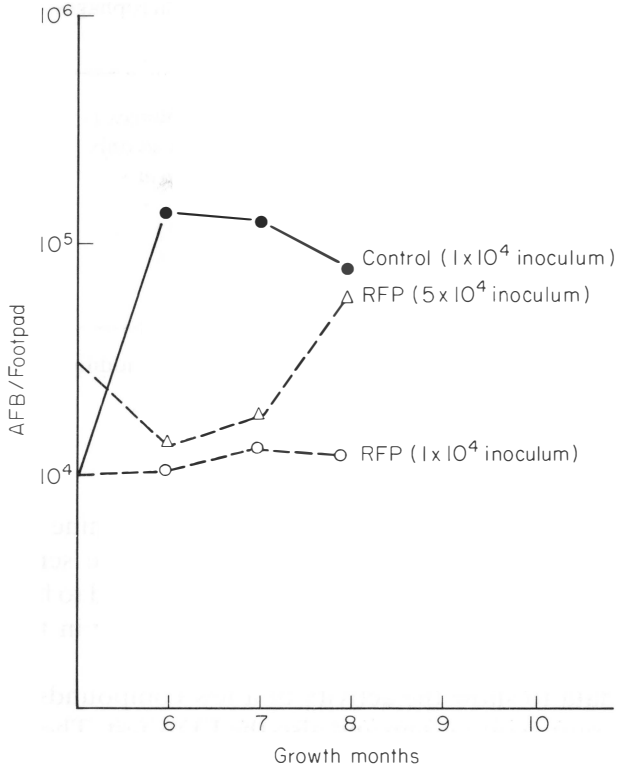

Figure 5. Loss of viability of *M. leprae* inside macrophages with rifampicin (RFP) (*in vivo* assessment). FDA viability: control, 30%; RFP, 1·14 μg/ml, 12·5%.

Table 12. Incorporation of ^{3}H-uracil by *M. leprae* (armadillo & human) inside macrophages (1×10^6) in presence of RFP.

	Ar_1	Ar_2	FMR 801*	FMR 805*	
Heat-killed *M. leprae*	1660	496	840	140	$p < 0{\cdot}01$ significant
Live *M. leprae* only	2950	1705	1140	994	$p < 0{\cdot}025$ significant
+ RFP (10 μg/ml) treated *M. leprae*	659	577	320	272	

* From patients.
Incorporation is expressed as dpm/1×10^6 *M. leprae*.

Table 13. Levels of removable sialic acid as n moles/10^6 macrophages in presence and absence of antileprosy drug, rifampicin.* (Macrophages from bacillary negative lepromatous leprosy patients.)

Expt. No.	Control macrophages	Macrophages + *M. leprae*	Macrophages + rifampicin only (5μg/ml)	Macrophages + rifampicin + *M. leprae*
1	25	10	22	25
2	28	8	25	26
3	30	12	28	30

* Rifampicin added to macrophages 48 hr before addition of *M. leprae*.

NEW COMPOUNDS—ANTI-M. LEPRAE ACTIVITY

If the above *in vitro* assay systems are useful to determine viability and drug sensitivity of *M. leprae* then new compounds could be screened for activity against *M. leprae* using these test systems. If they are found to be active against *M. leprae*, they should also show activity against *M. leprae* in the mouse footpad assay system.

We present data to show the activity of a few compounds against *M. leprae* using the Fc receptor assay system and also the FDA test. The compounds tested were Deoxyfructoserotonin, Brodimoprim (in combination with DDS), ciprofloxacin, methyoxy-2-Indole-derivative and Folate analogues, and K.130 in

Table 14. Percent EA rosetting of macrophages exposed to varying concentrations of deoxyfructoserotonin (DFS) in presence of *M. leprae*. (Percent EA rosetting (AV ± SD).)

DFS concentration (ng/ml)	Control macrophages only	Macrophages + live *M. leprae*	Macrophages + DFS	Macrophages + DFS *M. leprae*	Macrophages + heat-killed *M. leprae*
20	64 ± 7	37 ± 5	49 ± 3	41 ± 6	67 ± 6
50	57 ± 8	33 ± 4	45 ± 8	44 ± 8	58 ± 11
*70	62 ± 10	40 ± 14	46 ± 10	61 ± 10	63 ± 12
†80	62 ± 11	32 ± 16	51 ± 9	65 ± 16	62 ± 18
100	70 ± 18	35 ± 5	63 ± 20	78 ± 9	70 ± 11

* MIC of DFS.
† Concentration from which DFS could be immunomodulating.
Results are an average of 3 experiments.
Dose of *M. leprae*—5×10^6/Leighton tube.

Table 15. Percent EA rosetting by macrophages in presence of *M. leprae* and of varying concentrations of ciprofloxacin. (Percent EA rosetting Av ± SD).

Drug concentration (ng/ml)	Control macrophages only	Macrophages + live *M. leprae*	Macrophages + ciprofloxacin	Macrophages + ciprofloxacin + *M. leprae*	Macrophages + heat-killed *M. leprae*
20	61 ± 6	41 ± 8	46 ± 7	46 ± 3	65 ± 6
*50	61 ± 10	41 ± 16	47 ± 11	59 ± 7	61 ± 9
80	62 ± 9	40 ± 5	51 ± 8	65 ± 8	63 ± 14
100	63 ± 12	42 ± 9	56 ± 2	65 ± 6	59 ± 11

Results are an average of 3 expts at each concentration.
Dose of *M. leprae*—5×10^6/Leighton tube.
* MIC of ciprofloxacin—50 ng/ml.

combination with DDS (Tables 14, 15, 16, 17, 18). The data are presented and observations are mentioned in the legend under each table.

Among these compounds Deoxyfructoserotonin, Brodimoprim and K-130 (in combination with DDS) have been reported to be active on *M. leprae* in mouse footpad tests[13] (Gelber *et al.*, Dhople *et al.*, personal communications).

Discussion

The methods adopted for determining viability of *M. leprae* by us have been based on the behaviour of *M. leprae* and the host cell (macrophages) when they are together. None of the test systems depend on the necessity of multiplication of *M. leprae*. But they depend on the expression of the metabolic activity of *M. leprae* (FDA assay, uracil uptake) or changes introduced by the pathogen-host interaction in the host cell (Fc receptor changes, sialic acid change). It is clear from the data presented on the effect of drug on these parameters, that there is a definite MEC (minimum effective concentration) in all these cases. It was shown to be 0·028 μg/ml for DDS and 0·11 μg/ml rifampicin in Fc receptor assay system and 0·1 μg/ml. DDS and 0·17 μg/ml rifampicin in FDA assay system (Jagannathan & Mahadevan, 1986, Bhagaria & Mahadevan, under preparation). Thus it is clear that the phenomenon observed is basically an actual event which is controlled by the level of drug exposed to *M. leprae*.

Further it has been demonstrated when loss of viability of *M. leprae* was indicated in presence of the drug in the Fc receptor assay system (inability to reduce EA rosetting macrophages). Such *M. leprae* had also shown loss of

Table 16. Percentage of macrophages exhibiting Fc receptors in the presence of *M. leprae* (ML) with or without added dapsone (DDS) or brodimoprim (BDP) (means + SD from at least 3 experiments for each concentration).

Dosages of drugs added (μg/ml)		Control (macrophages only)	Treatment							Fractional inhibitory index
DDS	BDP		ML	BDP	DDS	DDS + BDP	DDS + ML	BDP + ML	BDP + DDS + ML	
0·022	—	70 ± 1	46 ± 7	—	55 ± 3	—	59 ± 3	—	—	—
0·028	—	75 ± 5	48 ± 6	—	65 ± 5	—	76 ± 5	—	—	—
—	5·7	66 ± 1	34 ± 2	45 ± 4	—	—	—	55 ± 1	—	—
—	7·1	70 ± 3	44 ± 3	55 ± 1	—	—	—	71 ± 5	—	—
0·014	2·8	69 ± 8	35 ± 2	56 ± 5	—	38 ± 2	—	60 ± 8	71 ± 11	0·9
0·007	1·4	78 ± 12	40 ± 7	58 ± 9	—	43 ± 4	—	50 ± 4	79 ± 12	0·45*
0·007	0·70	78 ± 3	42 ± 3	52 ± 1	—	64 ± 3	—	46 ± 3	78 ± 2	0·35*
0·007	0·35	74 ± 14	49 ± 4	50 ± 7	—	58 ± 7	—	49 ± 7	41 ± 8	—

* The most effective combinations of the two drugs.

Table 17. Percent EA rosetting of varying concentrations of K-119 (Indole-2-carboxylic acid) against *M. leprae*. (Percent EA rosetting (Av±SD).)

Drug concentration (ng/ml)	Control macrophages only	Macrophages + *M. leprae*	Macrophages + K-119	Macrophages + K-119 *M. leprae*	Macrophages + heat-killed *M. leprae*
20	65±9	36±8	46+8	49+14	65±9
50	59±8	32±9	36±4	38±7	58±6
*80	55±6	35±9	35±8	60±11	53±4
100	60±10	33±15	47±7	58±10	59±12

Results are an average of 3 experiments.
Dose of *M. leprae*—5×10^6/Leighton tube.
* MIC of K-119—80 ng/ml.

viability through their inability to hydrolyse FDA and consequent failure to exhibit green fluorescence. There is correlation between the two *in vitro* methods.

The fundamental correlation of loss of viability of *M. leprae* inside the macrophages in presence of drug and as determined by Fc receptor or FDA assay was the demonstration that such *M. leprae* showed no growth or at best poor growth in the mouse footpad. The *M. leprae* prepared in a similar way from macrophages without drug treatment showed good viability.

The routine procedure of using microscope and determining either fluorescence of the bacteria or rosetting macrophages was found to be a valid technique, since the observations obtained were correlated with spectrofluorimetric measurements or ^{125}I labelled antibody binding to Fc receptors in the respective tests mentioned above.

Thus it is very clear that we have demonstrated new *in vitro* assay systems capable of determining drug sensitivity and viability of *M. leprae* in less than 10–15 days. Consequently one should be able to determine the potentiality of anti-*M. leprae* nature of some new compounds. We have been able to do these kind of tests and have identified the following compounds as active against *M. leprae*: 1, Deoxyfructoserotonin; 2, Indole-2-methoxy compound; 3, Diflunisal (Merck, Sharp and Dohme); 4, Ciprofloxacin (Bayer); 5, Brodimoprim alone or in combination with DDS (Dr J K Seydel); and 6, K-130 (Dr J K Seydel).

Leprologists will accept efficacy of these drugs if they could be demonstrated to show activity against *M. leprae* infected in mice. The role of mouse metabolism preferentially inactivating the drug or poor pharmacokinetics in mice can lead to wrong data. But this is ignored.

Nevertheless if correlation comes, everybody is satisfied. Thus anti-*M. leprae* activity against *M. leprae* in mice has been shown for deoxyfructoserotonin,[13]

Table 18. Percent EA rosetting of macrophage with *M. leprae* exposed to varying concentrations of the drug K-130 along with DDS. (Percent EA rosetting (Av ± SD).)

Drug concentration		Control						
DDS (μg/ml)	K-130 (μg/ml)	(macrophages only)	Macrophages + *M. leprae*	Macrophages + K-130	Macrohages + K-130 + DDS	Macrophages + K-130 + *M. leprae*	Macrophages + K-130 + DDS + M. leprae	Macrophages + heat-killed *M. leprae*
0·007	0·01	65 ± 15	35 ± 7	—	42 ± 1	—	55 ± 14	64 ± 15
*0·007	0·02	80 ± 6	45 ± 11	—	61 ± 1	—	79 ± 6	78 ± 6
—	0·70	55 ± 8	31 ± 1	43 ± 15	—	39 ± 2	—	59 ± 7
†—	1·00	50 ± 1	31 ± 1	34 ± 3	—	50 ± 3	—	48 ± 2

* Amount of the drug K-130 for synergism with DDS—0·02 μg/ml.
† MIC of the drug K-130—1 μg/ml.
Dose of *M. leprae*—5×10^6/Leighton tube.
Data expressed as Av ± SD from a minimum number of 3 expts at each concentration.

Brodimoprim in combination with DDS (Gelber *et al.* personal communication), and K-130 (Dhople, personal communication).

We are now in the process of testing ciprofloxacin, diflunisal and Indole-2-methoxy compounds in the mouse footpad system in our laboratory.

It would be worthwhile to note, following the *in vitro* assay system in our laboratory, that DFS was identified as anti-*M. leprae*[2,7] and later confirmed in mouse footpad and is now in clinical trial in Bombay, India. This should be considered as a unique success in the drug development programme against leprosy. This success opens up vast potential for exploitation of the lead shown by the *in vitro* assay system developed in our laboratory.

The advantages of these *in vitro* assay systems are: (a) it is completed in less than 10–12 days; (b) *in vitro* MIC can be determined; (c) synergistic activity between two different drugs can also be established; and (d) static or cidal effect can be assessed. Among the drawbacks: (a) one needs at least 5–10 million *M. leprae* for each assay as compared to 1×10^4 in mouse footpad; and (b) as patients improve on drug therapy, viability goes down and thus to monitor the viability one has to use higher numbers of bacilli and this may lead to ambiguous information.

Nevertheless, we are in a position now to identify potential anti-*M. leprae* compounds much faster than we were 5 years ago. This is a definite advance in drug research in the area of leprosy control.

Acknowledgments

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Diflunisal and indole-2-carboxylic acid were identified as possible antileprotic agents and made available to us for testing as a result of an extensive study of tyrosinase inhibitors by our collaborators E G Beveridge, M Hooper and S K Yeap. *J Pharm Pharmac* 1985; **37**(Suppl.) 149P.

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Isolation of environment-derived *Mycobacterium leprae* from soil in Bombay

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It is well known that even in highly endemic areas, contact with a leprosy patient cannot be established as a source of infection in a considerable proportion of the new cases.[11] In a study covering Hawaii, Philippines, Indonesia and some countries in Africa, no contact could be established in 30–60% of the new patients.[1] Enna *et al.*[3] reported that in the continental United States only 25·8% of new leprosy cases had any known contact with leprosy patients.

Accordingly, attention is drawn to the possible existence of extra-human reservoirs and thus to extra-human sources of infection.[15] At the II International Leprosy Congress in 1909, Sand[17] postulated, on the basis of epidemiological observations, that leprosy is not transmitted by direct contact, but probably through some environmental medium such as soil. The finding in the 1970's of naturally acquired leprosy in the nine-banded armadillo[13] and in the Mangabey monkey[18] may be consistent with this hypothesis.

At the same time, evidence has accumulated supporting the potential existence of environmental sources of *M. leprae*, particularly in sphagnum vegetation.[8,5,14,4] However, until recently, the lack of sufficiently specific methods has hindered a thorough differentiation of the mycobacteria found in series of samples, obtained from various environmental sources.

In the present paper, a technique based on monoclonal antibodies specific for the major *M. leprae* phenolic glycolipid[19] was used, in addition to already established procedures, for further differentiation of the mycobacterial content of the samples.

Materials and methods

A total of 17 samples, 13 water samples and four soil samples, was randomly

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selected from six districts of the Bombay Leprosy Project area and in Kopary leprosy colony (Table 1), covering approximately 1 million of greater Bombay's total population of 8 million (Figure 1). In this area, about 40% of the population is living in slums.

The water samples were filtered with Antlia pneumatic pump SP 050/2 through membrane filter pore size 0·5 μm (Schleicher and Schüll, Dassel). The filters were kept in a refrigerator at 1°–4°C until processing. The soil samples were collected with sterile spoons into sterile plastic bags and refrigerated until processing. The membrane filters were homogenized in 3 ml of 0·1% sterile albumin solution, the soil samples were homogenized with equal volume of sterile distilled water allowed to stand for 10 min, the supernatant centri. 30 min at 5000 upm and resuspended in 3 ml of 0·1% albumin solution. From the fluids smears were prepared for counting of acid-fast bacilli.[10]

One millilitre of the fluid was treated with oxalic acid and $NaOH^2$ and inoculated on Löwenstein-Jensen, Middlebrook 7 H10 solid media in tubes and Middlebrook 7 H10 medium in plates. The plates were incubated for 6 weeks and the tubes 4 months at 31°C. The differentiation and taxonomic analysis of isolated mycobacteria were based on 46 properties.[7] Another portion of the fluid was inoculated, without treatment, subcutaneously (0·03 ml) into both hind footpads of 10 female white mice of the inbred strain NMRI-SPF (Lippische Versuchstierzucht, Extertal, W. Germany). The examination for acid-fast bacilli (AFB) was made at intervals of 6, 9, 12, 18 and 24 months.[10] AFB-positive homogenates from the footpads were examined for cultivable mycobacteria, and for further maintenance inoculated in sphagnum nutritive substrate.[9] When cultivable mycobacteria were excluded, 0·03 ml from the sphagnum substrate was inoculated in nu/nu mouse footpads (Gl. Bomholtgard, Ry, Denmark). From each sample at least 5 nu/nu mice were inoculated. The footpads were examined after 6 and 9 months. Homogenates of footpads containing more than 10^8 AFB were inoculated intravenously into four nine-banded armadillos. Noncultivable (in conventional media) AFB harvested from the footpads of nu/nu mice or sphagnum substrate were examined by dopaoxidase[16] and pyridine decoloration[12] tests. The two homogenates examined in this way contained 3·7 and $3{\cdot}5 \times 10^7$ AFB per ml, respectively, and were homogenized in 0·1% albumin and lyophilized without further purification. Lipid extracts were analysed by a chromatographic separation of the lipids followed by screening, using monoclonal antibodies specific for major *M. leprae* phenolic glycolipids.[19,20]

Results

The water samples contained counts of AFB ranging from 10^2 to 10^5 per ml and the soil samples contained in most cases 10^5 per g (Table 2). A total of eight

Table 1. Description of the water and soil samples collected in Bombay on 9 and 10 February 1981

Sample No.	Locality & sample description	pH	Water/soil Temp. °C	Sample size: filtered water/ml soil/g
1	Dharavi, water reservoir on the school roof, leaking water	7·11	26	600 ml
2	Dharavi, reservoir in the school, tap water	7·11	28	600 ml
3	Dharavi public, stored water-drum, road side	6·94	26	600 ml
4	Danda Kahr, drinking water, stored in copper pot	7·27	26	600 ml
5	Danda Kahr, bazar Galli, stored drinking water	6·93	30	600 ml
6	Kopari, leprosy colony Mulund, well water	7·37	26	800 ml
7	Kopari, leprosy colony Mulund, tap water from the well	7·24	26	800 ml
8	Kopari, leprosy colony Mulund, well water, another tap	7·28	26	1000 ml
9	Santa Cruz, Best Quarters, Chunabhatti drinking tap water, stored	7·14	26	1000 ml
10	Santa Cruz, Best Quarters, Chunabhatti soil sample, wet soil on the road side	7·40*	28	40 g
11	Santa Cruz, Best Quarters, Chunabhatti drained waste water along the road	ND†	ND	50 ml
12	Santa Cruz, Best Quarters, Chunabhatti, wet soil sample near waste water ditch	7·65*	30	75 g
13	Bharat Nagar (North) Bandra, stored tap water–washing water	7·03	28	800 ml
14	Bharat Nagar (Bandra) wet soil, house washing place	6·92*	29	85 g
15	BDD Chawl (Worli) tap water, stored in house	7·38	18	800 ml
16	BDD Chawl (Worli) dry soil sample from the street place	7·12*	30	45 g
17	Worli, Sea Corner, surface washing water flowing into the sea	7·04	29	800 ml

Samples No. 1,2,3,4,5,9,13 and 15: Public water supply of Great Bombay
* pH measured in water homogenates by processing in the laboratory
† Not determined

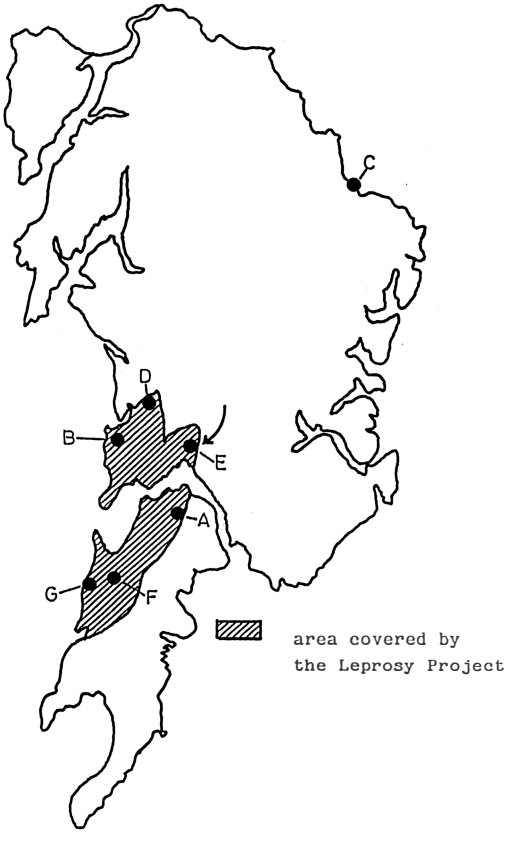

Figure 1. Greater Bombay and area covered by the Bombay Leprosy Project with the location of samples collected (A–G). Arrow indicates the finding of environment-derived *M. leprae*. Further explanation in Table 2.

samples contained strains of cultivable mycobacteria (Table 2). Some of those strains also survived in footpads. In one sample from soil (E), examination of footpads for AFB revealed repeatedly acid-fast bacilli which failed to grow on conventional media. The mean count per footpad of non-cultivable AFB increased from $4{\cdot}7 \times 10^5$ in 6 months to $8{\cdot}5 \times 10^6$ in 24 months. In nu/nu mice, after a mean inoculation of $8{\cdot}5 \times 10^3$ per footpad, an increase to a mean of $2{\cdot}4 \times 10^9$ was observed after 9 months. The swelling obtained after 6 months is shown in Figure 2. The test for specific phenolic glycolipid I was positive (Figure 3). Tests for dopaoxidase and pyridine decoloration were positive. In biological tests, nerve involvement in nude mice and systemic leprosy in one of the infected armadillos were found.

Table 2. Samples of water and soil collected in six slum districts and one leprosy colony with count of acid-fast bacilli (AFB) per ml and per g, respectively, and the species of mycobacteria isolated.

Slum District	Water sample		Soil sample		Species of mycobacteria
	Sample No.	AFB(x.10^3)/g	Sample No.	AFB(x.10^3)/g	
Dharavi (A)	1,2,3	0·83–42			*M. lactae* *M. thermorestible* *M. obuense* and *M. phlei*
Danda Kahr (B)	4,5	6·7–27			none
Kopari Leprosy Colony (C)	6,7,8	43–590			*M. thermorestible* *M. gordonae*
Chunabhatti (D)	9,11	2·4–80	10,12	4·8–320	none
Bharat Nagar (E)	13	0·14	14	420	*M. gadium* (water sample) *M. intracellulare* (soil sample)
BDD Chawl Worli (F)	15	7·0	16	650	*M. gilvum* (both samples)
Worli Sea Corner (G)	17	0·99			*M. gilvum* *M. sphagni*

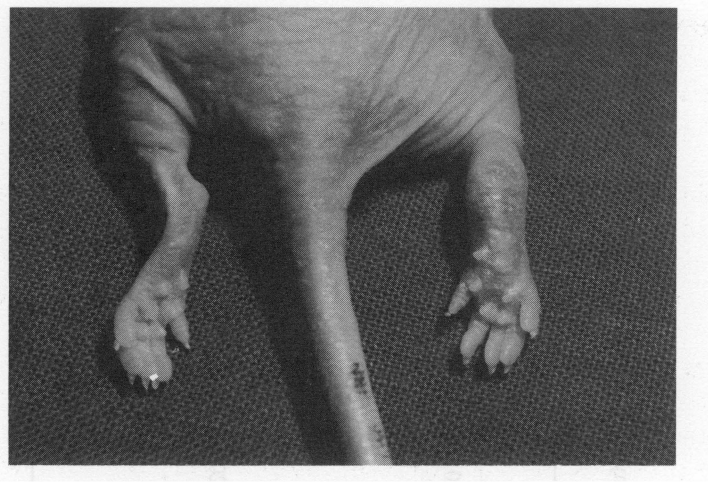

Figure 2. Swelling of the inoculated right footpad observed 6 months following infection (the left footpad served as a control).

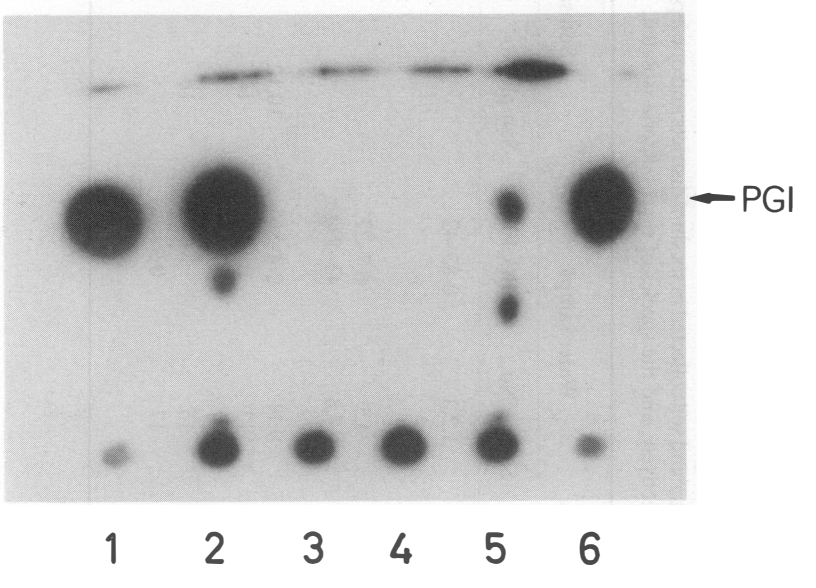

Figure 3. Phenolic glycolipid I (Ph I) in AFB harvested from inoculated footpads 6 months after inoculation (column 5). Column 1 and 6 phenolic glycolipid I (100 mg); column 2 positive leprosy serum; column 3 and 4 negative leprosy sera; column 5 footpad sample (E).

Discussion

The samples collected in Bombay 1981 have shown a variety of cultivable mycobacteria. Only in one sample could mycobacteria be found which did not

grow on conventional media, and which multiplied in normal and nu/nu mice like *M. leprae*. They cause leprosy in armadillo and nude mice. In this sample, antigens believed to be specific for *M. leprae* were detected. Apparently, this is the first reported isolation of environmentally-derived *M. leprae* in a leprosy endemic region.

The epidemiological implication of this finding would need further clarification. On one hand, the *M. leprae* strain identified may have been deposited at the spot some time ago from a leprosy patient by coughing or sneezing. In this case, the epidemiological relevance may be limited. On the other hand, the sample may represent a reservoir independent of deposit from human sources, and then the epidemiological implications would seem highly important.

However, the likelihood that the spot from where the sample was taken, was particularly infected from leprosy patients, was rather low; no patients were living in the nearest houses. Taking into consideration the epidemiological indications already mentioned, the finding seems to support the concept of environmental sources of infection in leprosy. Clarification of the issue needs further cooperative microbiological and epidemiological studies based on more simple but still reliable methods for the identification of *M. leprae*.

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Investigations into the cultivation of *Mycobacterium leprae*. A multifactorial approach

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Introduction

Evidence is accumulating that *Mycobacterium leprae* is probably a microbe-dependent microbe.[10–13] The first report that multiplication of *M. leprae* was promoted by a growth factor of mycobacterial origin was proposed by Twort[19] in 1910, two years before the discovery of the cultivation of 'Johne's bacilli' in media enriched with heat-killed mycobacteria.[20] The growth factor was identified 37 years later as mycobactin.[4] It became evident that mycobacteria necessitate two distinct iron transport compounds: exochelins for extracellular iron acquisition and mycobactins for cell-wall iron transport and intracellular iron storage.[14,15]

In attempting *in vitro* cultivation of *M. leprae*, the results of Twort[19] remained unnoticed until Hanks[6] adopted 'the mycobactin-requiring *M. paratuberculosis* as a model toward the cultivation of *M. leprae*'. When Ishaque and Kato[7] reported the presence of a cytochrome system in *M. leprae*, iron was recognized as an essential element for *M. leprae*. Consequently, as for all mycobacteria, the iron transport compounds are integral mediators for the functioning of the iron-dependent cytochromes, electron transport and energy metabolism of *M. leprae*.

Hall, Wheeler & Ratledge[5] have shown that exochelins from *M. neoaurum* mediate extracellular iron uptake into *M. leprae*. Due to methodological difficulties, it was not evident whether *M. leprae* could produce its own exochelins. *M. leprae* is, however, deficient in mycobactin, as reported by Kato.[11]

The above data, coupled with the ever-increasing number of reports on the cultivation of leprosy-derived mycobacteria from *M. leprae*-infected human and armadillo tissues, prompted Kato to propose that *M. leprae* was probably a microbe-dependent microorganism[10,11] This concept (Table 1) implies that the cultivable leprosy-derived mycobacteria are an integral part of the pathology, by providing the iron transporting compounds for *M. leprae*.[10–13]

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Table 1. Data leading to the concept that *M. leprae* might be a microbe-dependent microbe

Year	Authors	Pertinent data
1910	Twort[19]	Cultivation of *M. leprae* on media enriched with killed 'Tubercle bacilli'
1912	Twort and Ingram[20]	Cultivation of Johne's bacilli. Concept of a growth factor for mycobacteria
1949	Francis *et al.*[4]	Growth factor identified as mycobactin
1966	Hanks[6]	Microbe dependent mycobacteria: models for growing *M. leprae*
1975	Macham & Ratledge[14]	Iron acquisition of mycobacteria by exochelins
1975	Macham *et al.*[15]	Necessity of two distinct iron transport compounds for mycobacteria: exochelins and mycobactin
1977	Ishaque *et al.*[7]	Cytochrome system present in *M. leprae*: iron is essential
1983	Hall *et al.*[5]	Exochelin mediated iron uptake into *M. leprae*
1873–1986	See[9–11]	Frequent cultivation of mycobacteria from *M. leprae* infected tissues
1984	Kato[10]	Leprosy derived mycobacteria, probably etiological cofactors in leprosy
1985	Kato[1–13]	Absence of mycobactin in *M. leprae*: probably a microbe dependent microbe

Exochelins did not act as a growth factor for *M. leprae* in the experiments of Hall & Wheeler.[5] In full agreement with Hall *et al*,[5] the cultivation of *M. leprae* must be considered 'a multifactorial problem'. In searching for such multifactorial, cooperating factors, contributing to the *in vivo* and *in vitro* growth of *M. leprae*, some data are already available and summarized in Table 2.

Maximal endogenous respiration by host-grown *M. leprae* was registered at pH 5·8.[7] This H-ion concentration, maintained by a PO_4 buffer, is proposed for a prospective culture medium. Ferric ions will be necessary for the functioning of the cytochromes.[7] Endogenous respiration of *M. leprae* was stimulated by sulphydril compounds.[7] For this purpose Na thioglycolate is an ideal candidate in the medium.[1] Yeast extract also stimulated respiration and ATP formation. A chemically well defined, potent growth promoting ingredient in yeast extract (thioctic acid)[16,19] is a promising factor for growing *M. leprae*. Since exochelins mediate iron uptake for *M. leprae*[5] and *M. leprae* does not produce mycobactin,[11] it is logical to incorporate both exochelins and mycobactins in a prospective culture medium for *M. leprae*. It is obvious that exochelins and mycobactins produced by leprosy-derived mycobacteria (LDM) should be used.

Ishaque & Kato[7] found the cytochromes in a reduced state in *M. leprae*, suggesting reduced O_2 requirements for *M. leprae* and probably toxic effects of

Table 2. Data pertinent to the formulation of prospective multifactorial culture media for *M. leprae*

Data on *M. leprae*	Proposed constituents in media
Ishaque & Kato[8]—Optimal endogenous respiration pH 5·8. Cytochrome system present. Iron needed. Respiration stimulated by SH compound. ATP formation stimulated by yeast extract	M/15 PO_4 buffer pH 5·8, ferric ion, Na thioglycolate, and lipoic (thioctic) acid
Hall *et al.*[5]—Iron uptake mediated by exochelins	Exochelins (for iron uptake) from leprosy derived mycobacteria
Kato[11]—Absence of mycobactin in *M. leprae*	Mycobactins (for intracellular iron transport/storage) from leprosy-derived mycobacteria
Ishaque *et al.*[7]—Cytochromes present in reduced state	Reduced O_2 tension in media
Campbell[2]—Low O_2 concentration in tissues	Microaerophilic conditions
Macham *et al.*[15]	Exochelins and mycobactin in filtered spent Tween 80 media

high O_2 tension. Oxygen concentration is low in tissues.[2,18] Therefore, microaerophilic conditions in media might contribute to growth of *M. leprae.*

The above multifactorial conditions will be considered in proposing media for cultivation trials of *M. leprae.*

Materials and methods

MICROORGANISMS

Mycobacterium leprae cell suspensions were obtained aseptically from spleen or liver of four *M. leprae*-infected armadillos. Partially purified cell suspensions were obtained by differential centrifugation, and were treated with 2% NaOH for 20 min. Following neutralization with HCl, cells were washed with M/15 phosphate buffer pH 6·5 and re-suspended in the liquid medium to obtain approximately 10^7–10^8 acid-fast rods/ml. Bacilli were sent *via* air mail in this medium. Cell suspensions arrived in Montreal in 8–10 days.

M. intracellulare (LDM) (serotype 19) was isolated from an *M. leprae*-infected armadillo.

M. phlei and *M. scrofulaceum* (H1-75) (both LDM) were isolated from lepromata of lepromatous leprosy patients.

M. avium-intracellulare (Lufu), an environmental isolate by F. Portaels.

M. paratuberculosis ATCC 19.698

EXOCHELIN (E) AND EXOCHELIN–MYCOBACTIN (E–M) RICH FILTRATES

Two LDM, *M. intracellulare* and *M. phlei*, were grown respectively in 500-ml flasks containing each 200-ml iron-deficient glycerol-asparagin (Sauton) media. *M. intracellulare* was cultivated for 20 days, *M. phlei* for 10 days at 34°C. Cultures were autoclaved for 45 min and filtered while hot on filter paper. The filtrates were collected and served for the exochelin media.

The filtrates were designated as: IE, E filtrate of *M. intracellulare*; and PE, E filtrate of *M. phlei*.

The two LDM were also grown in the iron-deficient Sauton media containing 1% Tween 80, resulting in the presence of mycobactins—in addition to exochelins in the filtrate according to Macham *et al.*[15] The filtrates were designated as: IEM, E/M filtrate of *M. intracellulare*; and PEM, E/M filtrate of *M. phlei*.

Exochelins and mycobactins were not extracted and purified from the filtrates. When adjusted to pH 6·5 and autoclaved, the filtrates promoted growth of the mycobactin-dependent *M. paratuberculosis* ATCC 19.698.

THE MEDIA

In order to assure the hypothetical multifactorial growth requirements for *M. leprae*, data of Table 2 were taken into consideration. The proposed culture media thus contained the respiration-promoting Na-thioglycolate and thioctic acid as oxidizable substrates for energy generation in appropriate inorganic salts dissolved in the filtrates containing mycobactin–exochelin.

To each litre of the filtrates (IE, PE, IEM, PEM) respectively was added $(NH_4)_2SO_4$ 2 g, Na-thioglycolate 1 g, $MgSO_4$ 0·1 g, thioctic acid 0·1 g, and ferric ammonium citrate 0·05 g.

The solutions were then adjusted to pH 5·8 with KH_2PO_4. In each of the 25-ml screw-cap tubes, 12-ml aliquots of the media were distributed and sterilized for 30

Table 3. Schematic presentation: growth of mycobacteria from *M. leprae*-infected tissues in multifactorial media enriched with factors from leprosy-derived mycobacteria (LDM)

Medium: Souton: S Basal: B	Factor Donors: LDM	Factors in media		Growth in cultures	
		Exochelin	Mycobactin	Primary	Subcultures
S: IE	*M. intracell*	+		Limited 3/4	No
S: IEM		+	+	Positive 3/4	Positive 3/4
S: PE	*M.*	+		Limited 4/4	No
S: PEM	*Phlei*	+	+	Positive 3/4	Positive 3/4

min in an autoclave. The obtained media were designated as S:IE, S:IEM, S:PE and S:PEM, thus indicating their composition on Table 3.

CULTIVATION AND ESTIMATION OF GROWTH

Enrichment and adaptation

One millilitre of the host-grown *M. leprae* suspension was transferred into each of the tubes containing 20-ml media. A one-week incubation period served to adapt the host-grown cells to the *in vitro* conditions and substrates of the medium.

Primary cultures

The 7th day, cultures were shaken for 5–10 s with a Vortex Junior apparatus and transferred 1:10 into the homologue media. The cultures were incubated at 34°C.

Quantitative estimation of growth was not feasible in these primary cultures, due to the uneven clumping of the bacilli. Increased turbidity did not necessarily reflect cell multiplication, due to co-precipitation and agglomeration of bacilli with host components.

Subcultures

When microscopic examination showed a considerable increase of bacterial masses, the cultures were again transferred 1:10 into the homologue medium. This was usually effected from 4- to 6-week-old primary cultures into the subcultures.

Growth kinetics were estimated in the subcultures. Increased turbidity of the cultures was registered by nephelometric measurements, when preliminary tests indicated that cell concentration and turbidity were of linear relationship during the exponential growth phase in the subcultures. Growth kinetics as expressed in changes of nephelometric readings are only relative values, not necessarily reflecting the real rate of multiplication. This is due to the nature of the media, containing the water-insoluble mycobactins in a labile colloidal system, thus interfering with the quantum of measurable transmitted light in the optical system.

Cultures were transferred regularly at 8- to 12-week intervals.

Microscopic examination

One 4-mm loopful of the cultures was gently spread on a 1-cm diameter field on siliconized slides and dried at room temperature for 24 hr. This technique permitted adhesion to the slides during staining and the preservation of growth morphology (cords, spirals, clumps) during growth. Following treatment with

10% fresh periodic acid at 80° for 2 min regular Ziehl–Neelsen staining was performed.

Pyridine extractability of the *in vitro*-grown cells and of the bacilli recovered from the footpads was tested according to Fisher & Barksdale.[3]

Mouse footpad inoculation

Twenty Swiss albino mice were each inoculated with 10^4 acid-fast bacilli in the left hind footpad. Four mice were killed at 50-day intervals and the number of bacilli per footpad was registered.

IN VITRO DRUG SENSITIVITY OF LDM AND *M. INTRACELLULARE (LUFU)*

Three strains of LDM and *M. Lufu* were tested for drug sensitivity on Middlebrooke and Cohn's 7H10 medium. Drugs were incorporated into the media before solidification. Colonies were counted after 21 days of incubation. Results are expressed as resistance (R) or sensitivity (S) to the concentration of drugs recorded in Table 4.

The three strains, cultivated in S:IEM media from *M. leprae*-infected armadillos, were tested for drug sensitivity in S:IEM liquid media. Resistance (R) or sensitivity (S) was measured nephelometrically after 60 days of incubation.

Table 4. Schematic presentation: effects of drugs on *M. leprae* in mice and man, on leprosy derived mycobacteria and *M. intracellulare lufu*

	SM	INH	PAS	TBI	EMB	CY	ETH	DDS	CFZ	RFP
M. leprae/mice	S	S	S	S/R	R	S/R	S	S	S	S
man	R	R	R	R		R		S	S	S
(*Experientia* **34:** 1322, 1978)										
In vitro mcg/ml	10	5	10	4	16	40	40	25	20	1
M. intracell. (LDM)	R	R	R	R	R	R	S	S	S	S
M. scroful. (LDM)	R	R	R	R	R	R	S	S	S	S
M. intracell. (Lufu)	R	R	R	S/R	R	R	S	S	S	S
M. leprae? In B:IEM	R	R	R	R	R	R	R	S	S	S

SM, streptomycin; INH, isonicotinic acid hydrazide; PAS, para-aminosalicylic acid; TBI, 4-acetemino benzaldehyde thiosemicarbazone; EMB, ethambutol; CY, cycloserine; ETH, ethionamide; DDS, 4-4-diaminodiphenylsulfone; CFZ, clofazimine; RFP, rifampicin. S, sensitive; R, resistant.

Results

Multiplication of acid-fast cells was not investigated during shipment, the period of enrichment and adaptation, or in the primary cultures. These time-consuming measurements were neglected, because only cultivation in successive subcultures can be considered successful *in vitro* growth. Results in Tables 3 and 4 show that this was indeed achieved in the multifactorial media containing exochelins and mycobactins produced by any of the two LDM: *M. intracellulare* or *M. phlei*.

Four suspensions of host-grown *M. leprae* cells from armadillos were inoculated into the media shown in Table 3. Out of the four specimens, positive cultures were obtained in three trials. The three cultures had similar growth characteristics; only the latency period of growth was different, probably due to differences in the number of viable units in the inocula. For this reason cultivation of only one of the three strains will be described as a representative culture out of the three now maintained in subcultures.

In the primary cultures, there was seemingly a considerable increase in turbidity in 2–4 weeks, as well as an increase in bacterial mass microscopically. Without quantification, however, this was not considered as growth. Following 4 weeks of incubation, the cultures were transferred into the homologue media. Immediately the base line of turbidity and microscopic characteristics were registered.

The mycobactin-dependent strain of *M. paratuberculosis* grows abundantly in the Sauton medium enriched with exochelin and mycobactin. This is evidence that the media indeed contained the iron transporting factors.

Multiplication of mycobacteria in the media inoculated with *M. leprae* are shown schematically in Table 3 and semiquantitatively in Table 5. Growth was

Table 5. Nephelometric readings of the fourth subculture of mycobacteria grown in multifactorial media inoculated with *M. leprae* (armadillo)

			Nephelometric values incubation time in days				
Cultures	Media	Opalescence	0	10	30	60	80
None	S: PE	–	12			16	
	S: PEM	++++	36			46	
M. leprae	S: PE		28			32	
	S: PEM		58			186	

Heat-killed *M. leprae*. No changes in nephelometric readings. *M. paratuberculosis*. Positive cultures in the PEM media.

not observed in any of the multifactorial media if enriched with exochelins alone. However, positive cultures were obtained if media were enriched with both factors: exochelin plus mycobactin. Both LDM provided active growth-promoting exochelins and mycobactins.

Nephelometric measurements and microscopic examination left no doubt that there was a definite multiplication in the first and the following subcultures. The estimated latency period of growth is about 10–16 days, and the division time during the exponential phase is probably close to 8–12 days.

Data in Table 5 are presented as evidence of growth in the fourth subculture of one of the strains in the various media. The multiplication of acid-fast bacilli in the media was so obvious that counting of bacilli was not necessary. Turbidity measurements were made nephelometrically to visualize growth in the *M. leprae*-inoculated multifactorial media.

Growth occurred as a flocculant sediment which was easily, but not completely, homogenized by shaking for 5 sec with the Vortex apparatus. Culture smears showed a tendency of the cells to clump together. When sedimented for 1 min on siliconized slides, it became evident that the flocculant growth consisted of small, and often extremely large, clumps or cordlike arrangements of the relatively large acid-fast rods. Acid fastness of the bacilli was extracted by pyridine.

DRUG SENSITIVITY OF LDM

Data presented in Table 4 show that, while all the tested antituberculous drugs except ethambutol have a complete or partial suppressing effect on *M. leprae* in the footpads of mice, only DDS and clofazimine have a slow and rifampicin a fast and potent therapeutic effect on leprosy in man.

Results show that only those drugs which show established clinical efficiency in human leprosy have inhibiting effects on the growth of LDM and *M. intracellulare (Lufu)*.

Rifampicin, the most potent drug in human leprosy, with a minimal bactericidal concentration of 1 to 1 μg ml in the serum of mice, inhibited completely the growth of our cultures in a dose as low as 1 μg ml. A relatively high concentration of 25 μg ml of DDS and 20 μg clofazimine was necessary to inhibit the growth of the same cultures *in vitro*. The minimum inhibitory concentration of DDS is 0·01–0·03 μg ml in the serum of mice against *M. leprae* in the footpad. DDS, however, is a slow-acting drug in man and has only bacteriostatic effects. All the strains isolated from human and armadillo leprous tissues showed resistance against all the other anti-tuberculous drugs tested.

Animal inoculations

The fourth subculture grown in PEM medium was inoculated into the footpads of

mice. The growth of the bacilli in the mouse footpad was somewhat faster than the usual pattern obtained following injection of host-grown *M. leprae* (probably due to the presence of mycobactin). When 10^4 acid-fast bacilli were injected per footpad, an average of 2×10^6 cells were recovered in 160 days. Bacilli recovered from the footpads lost their acid fastness after pyridine extraction, but remained Gram positive.

Discussion

In addition to previously published data,[9–13] the following experimental evidence is offered as indicative that *M. leprae* might indeed be a microbe-dependent microorganism and that the obtained cultures are probably identical to *M. leprae* or cultures mixed with LDM.

Media were inoculated with authentic host-grown *M. leprae* cells.

Cells grow in special multifactorial media.

Growth occurred under physical conditions optimal for the endogenous respiration of *M. leprae*: pH 5·8 at 34°C.[8]

Both known respiratory stimulants of *M. leprae*,[8] a SH compound, Na thioglycolate, and a yeast extract growth factor, thioctic acid,[1,21] were efficient supplements in media in which growth was obtained.

Positive cultures were obtained in media containing both exochelins and mycobactin[15] supplied by a LDM: *M. phlei*.

Cultures did not grow on Löwenstein or in Dubos media, but in the footpads of mice produced the disease similar to that caused by host-grown *M. leprae*.

Strong acid fastness of the cells was eliminated by pyridine.

Cultures show a resistance pattern to antibacterial agents, comparable to drug sensitivity of *M. leprae* in man.

The frequent presence of LDM in *M. leprae*-infected tissues is well documented. LDM are hard to grow and are present in extremely small numbers in leprotic tissues. It is highly probable that more leprosy infected hosts harbour LDM than are reported in the literature. Little attention was paid to the possibility that LDM were present at anatomical locations remote from the *M. leprae*-infected sites. It is known that secondary strains of mycobacteria were cultivable from organs remote from the tissues infected with *M. paratuberculosis* in Johne's disease of cattle. This microbe-dependent microorganism is dependent on growth factors of the secondary mycobacteria from a remote organ. A similar mechanism in leprosy remains to be investigated.

It was previously proposed[10] that attempts to cultivate *M. leprae* must be focused on media highly selective for *M. leprae*, without promoting the growth of the accompanying cultivable mycobacteria. This problem is complicated by the fact that *M. leprae* is the slowest of the slow growers and even the slow-growing secondary species will overgrow *M. leprae* in nonselective media. It is predictable

that *M. leprae* might also be grown and isolated from nonselective semi-solid media or, when the secondary LDM are present, at sites remote from the *M. leprae*-infected lesion of which cultivation is attempted.

It did not become evident whether cultures were pure or of a mixed nature.

The presented results support the view of Hall *et al.*[5] that to grow '*M. leprae* is probably a multifactorial problem'. In a multifactorial medium several components might be replaced, but exochelins and mycobactin are a *sine qua non* of success. The findings of Macham *et al.*[15] were of practical importance in obtaining exochelin and mycobactin-enriched media using Tween 80 containing spent cultures.

The *in vitro* drug sensitivity of LDM merits special attention. Since the *in vitro* cultivation of *M. leprae* remains to be proven, no *in vitro* pharmacological model is available for screening substances with prospective antileprosy effect. The only reliable test subject for screening drugs for activity against leprosy is the human lepromatous leprosy patient. LDM, however, presents a characteristic resistance pattern to antibacterial agents that is comparable to the drug sensitivity of *M. leprae* in man. This knowledge leads to the proposal of an *in vitro* method using LDM for screening drugs against leprosy.

Results presented in this communication have furnished further indirect but strong experimental evidence that *M. leprae* might be a microbe-dependent microorganism. This concept is supported by the evidence that multiplication of mycobacteria occurred only in *M. leprae*-inoculated multifactorial media which contained growth factors produced by LDM. The question thus arises as to whether antileprosy therapy should be targeted against *M. leprae* or against the donors of growth factors: the LDM. The results presented clearly show that LDM are highly sensitive to drugs which show therapeutical effects in lepromatous leprosy patients. I propose that the curative effects of antileprosy agents act by eliminating the secondary mycobacteria (LDM) which seem to be an integral part of the pathology as etiological cofactors in leprosy.

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SESSION III. DEVELOPMENTS AND FUTURE ASPECTS

Chairman: ENNO FREERKSEN (FRG)

Lepr Rev (1986) **57,** Supplement 3, 223–234

Recent developments in the field of multidrug therapy and future research in chemotherapy of leprosy

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The discovery of rifampicin in the late sixties together with the increasing prevalence of dapsone resistance were decisive factors in questioning the value of the traditional dapsone monotherapy for the treatment of leprosy. As rifampicin demonstrated a strong bactericidal activity against *Mycobacterium leprae*, it became an obligatory component of the treatment of leprosy during the decade 1970–1980, at least when its cost was not prohibitive. Then it was progressively understood that leprosy, a mycobacterial disease with a large bacillary population like tuberculosis, had to be treated like tuberculosis with a combination of drugs.[1–4] Actually to be a success, chemotherapy for leprosy as well as for tuberculosis should be capable of preventing the selection of drug-resistant organisms (mutants) and killing as many as possible of the drug-sensitive organisms.[5] When the selection of drug-resistant mutants is prevented, no failures during chemotherapy and no relapses after stopping chemotherapy due to acquired drug resistance will occur. When all or nearly all drug-sensitive organisms are killed, no relapse or a limited number of relapses with sensitive organisms will occur after stopping chemotherapy.

The aim of this paper is to summarize what is known about the microbial population present in multibacillary cases of leprosy and the response of these microbial populations to the drugs administered during the course of chemotherapy. Finally a series of leads for future research in chemotherapy of leprosy will be proposed.

1 The microbial population in multibacillary leprosy

It is assumed[6] that the maximum number of acid-fast bacilli (AFB) in the majority of multibacillary cases of leprosy is not more than 10^{11}, i.e. 11 decimal logarithms (log) AFB. This assumption fits well with the calculations made from the number of AFB in the biopsies taken from multibacillary cases of leprosy. As shown in

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Table 1, among 320 biopsies received in our laboratory for mouse inoculation from January 1980 to December 1985, 75% contained between 5 and 7 log AFB per mg of tissue, that is 8–10 log per gram, which means approximately 10–12 log bacilli per patient. Among the total 11 log AFB that are present before the start of chemotherapy as few as 1–5% are capable of growing in the footpad of mice and thus are considered viable. Therefore, the mean size of the viable bacillary population of a patient with multibacillary leprosy may be estimated to be about 9 log.

From the chemotherapeutic point of view the viable bacillary population is composed of various subpopulations, a large one of drug-sensitive organisms and small populations of drug-resistant organisms, each of them consisting of mutants resistant to one definite drug. By analogy with what is known about *M. tuberculosis*[7–10] and only by analogy because no direct evidence is available for the time being about *M. leprae*, one may estimate the mean proportion of resistant mutants within a wild strain of *M. leprae* to be 10^{-7} (-7 log) for rifampicin (RMP) and -6 log for dapsone (DDS), clofazimine (CLO) or a thionamide (TH), ethionamide (ETH), or prothionamide (PTH). Thus besides the 9 log subpopulation of organisms sensitive to all drugs, there should be a small subpopulation of 2 log RMP-resistant mutants and several subpopulations of 3 log DDS, CLO and TH resistant mutants (Figure 1).

The outcome of the different subpopulations differs according to the type of chemotherapy prescribed. If monotherapy (for example dapsone or rifampicin alone) is prescribed to treat a lepromatous patient the drug-sensitive organisms will be progressively eliminated whereas the subpopulation of mutants resistant to the prescribed drug will survive, be selected and be responsible for late relapses with drug-resistant organisms. If multidrug therapy is prescribed, there will be no survival and therefore no selection of drug-resistant mutants because each drug will be active against the mutants resistant to the other drugs. However, to ascertain that no drug-resistant mutants will be selected multidrug therapy should be given until all drug-resistant mutants present at the start of treatment are killed. Moreover, as new drug-resistant mutants can arise from the 9 log sensitive subpopulation, multidrug therapy should be given until this subpopulation is reduced below 6 log, a level under which the probability for a new drug-resistant mutant to occur is extremely low. In summary, to prevent the selection of drug-resistant mutants (in other words, to prevent acquired drug resistance), multidrug therapy should be given as long as drug-resistant mutants present at the start of therapy are not killed and as long as the size of the sensitive bacillary population is not strongly reduced. These are the two objectives of the initial phase of multidrug therapy (Figure 1), the duration of which is still unknown as we will see later on.

Then it remains to take care of the relatively limited number of sensitive organisms that have survived the initial phase of chemotherapy. At this stage there is no longer a risk of selecting drug-resistant mutants, thus combined

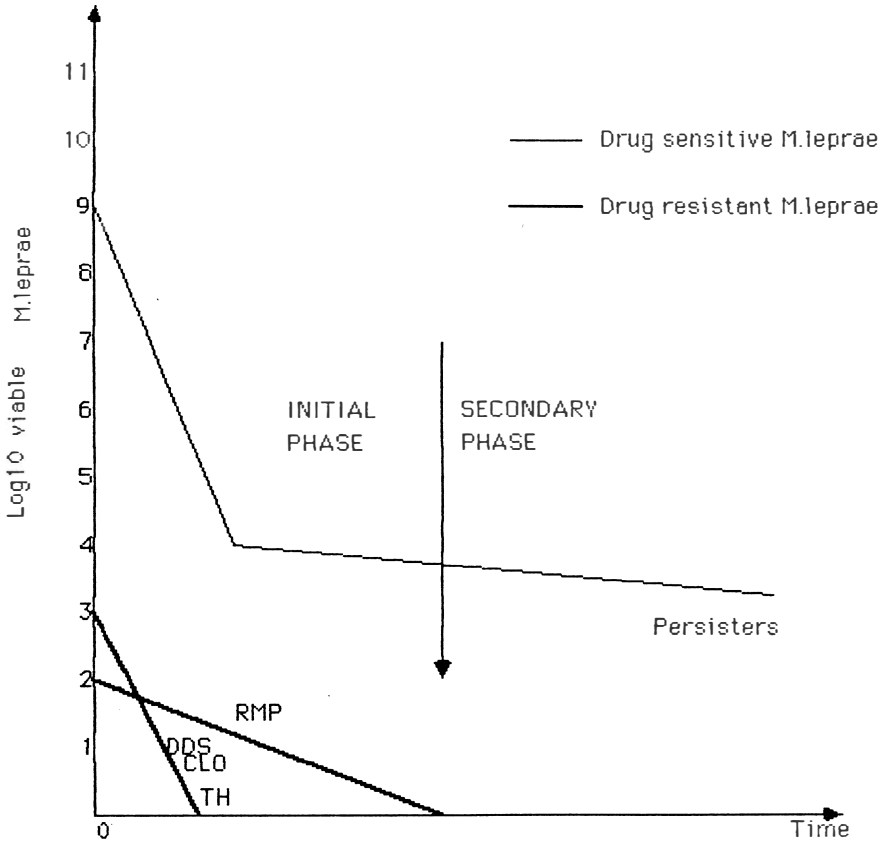

Figure 1. The outcome of *M. leprae* subpopulations during multidrug chemotherapy of multibacillary leprosy. (RMP, rifampicin; DDS, dapsone; CLO, clofazimine; TH, thionamide).

chemotherapy is no longer required. Drug therapy should be used to kill the remaining organisms, or at least to keep them in a dormant state so that the clearing mechanisms of the host can eliminate them progressively. If multidrug therapy is given at this stage it is because the combination of drugs has demonstrated (or is thought to have) a synergistic activity against *M. leprae*. One important question concerning the secondary phase of chemotherapy is to know how long this secondary phase should be continued? The evident answer is until a period of time such that there will be no relapse at all or an acceptable proportion of relapses ($\leq 5\%$) after stopping treatment.

Therefore, it is important to examine successively what is known about the initial phase and the secondary phase of chemotherapy and in which directions the future investigations in leprosy chemotherapy should go.

2 The initial phase of chemotherapy for leprosy

At present, a number of data[11–14] are available about the initial phase of chemotherapy for leprosy with rifampicin. First of all it was demonstrated that biopsies taken from patients having received a single dose of 600 mg rifampicin failed usually to give growth of *M. leprae* in the footpad of mice, whereas it took 3–6 months of daily treatment to obtain the same results with either dapsone alone, or clofazimine or ethionamide.[15–18] The extremely rapid initial killing of *M. leprae* by rifampicin has been measured in the recent Bamako–Chingleput study[19] carried out within the THELEP activities. After three months of chemotherapy with either daily 600 mg rifampicin, 100 mg dapsone and 100 mg clofazimine or

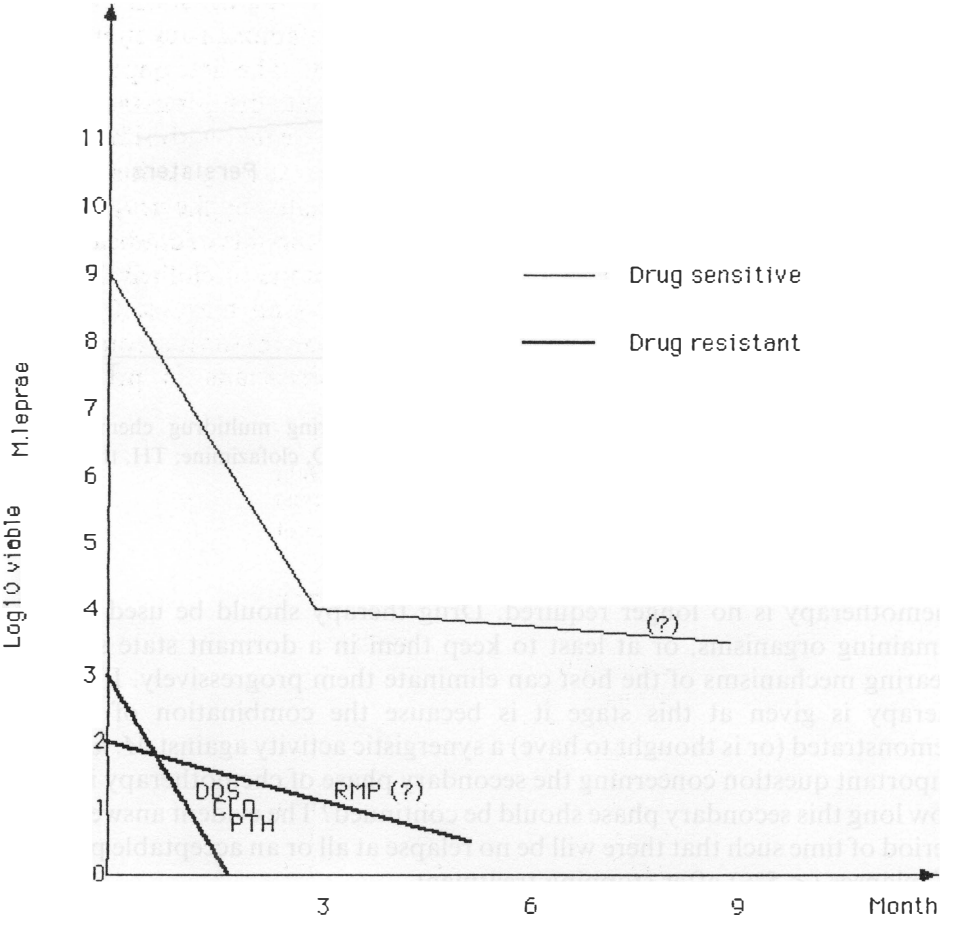

Figure 2. Known and unknown data on the outcome of *M. leprae* subpopulations under combined chemotherapy with rifampicin.

weekly 600 mg rifampicin plus daily dapsone and clofazimine or daily dapsone supplemented by an initial single dose of 1500 mg rifampicin, the proportion of AFB capable of multiplying in the hind footpad of thymectomized-irradiated (TR) mice was in the range of −7 log. Because the proportion of AFB that were able to grow in mice were not different among patients in the 3rd month of chemotherapy who received daily 600 mg rifampicin and patients who received only a single initial dose of 1500 mg rifampicin, one is led to assume that the initial killing resulted mainly from the first dose of rifampicin. If 1% of the 11-log AFB present at the start of treatment were viable, then a few days after the start of chemotherapy rifampicin should have killed 5 log of the rifampicin sensitive *M. leprae*. These include not only the majority of the largest subpopulation of fully sensitive bacilli but also all of the two 3-log subpopulations of dapsone- and clofazimine-resistant mutants which are by definition susceptible to rifampicin.

After the initial dose of rifampicin the only surviving organisms should be about 4 log rifampicin-sensitive bacilli and the 2 log rifampicin-resistant mutants that were already present before beginning treatment. The first population of 4 log rifampicin-sensitive bacilli is too limited to give rise to drug-resistant mutants. Therefore, when multibacillary cases of leprosy are treated with rifampicin the only risk of acquired resistance comes from the subpopulation of 2 log rifampicin-resistant mutants. These have to be eliminated by the drug(s) given in combination. In theory only, a single drug should be capable of eradicating these 2 log rifampicin-resistant mutants, for example dapsone or clofazimine. But in practice it is safer to give two drugs for the following reasons: (i) the high prevalence of primary and acquired dapsone resistance; (ii) the noncompliance of patients to take the prescribed drugs and of physicians to prescribe the

Table 1. Number of acid-fast bacilli in biopsies received in Paris between 1980 and 1985 from multibacillary cases of leprosy

Log_{10} AFB per mg	Biopsies	
	N°	%
less than 3	34	10·6
3 to 4	5	1·5
4 to 5	38	11·9
5 to 6	125	39·1
6 to 7	117	36·6
7 and more	1	0·3
Total	320	100

recommended regimens; and (iii) the relatively low killing activity of drugs other than rifampicin. Although the first two reasons are of utmost importance we are not going to concentrate on them. It is enough to say that it is not safe to rely entirely on dapsone alone to kill the RMP resistant mutants, especially in previously dapsone treated patients. Rather, dapsone should be given with another drug, either a thionamide or clofazimine, or both. In field practice many people are now reluctant to use a thionamide at least in some areas because of its liver toxicity, especially in combination with rifampicin.[20–23] Thus one should mainly rely upon the combination dapsone–clofazimine to get rid of the RMP-resistant mutants.

One can wonder whether all of the above reasoning is based upon theoretical speculation or realistic assumptions. Actually, if we consider (Table 2) the results of drug sensitivity testing of *M. leprae* isolated in our laboratory between 1980 and 1984 it is clear that the risk of drug resistance is a reality. Biopsies of 69 patients mainly from Caribbean Islands (Martinique, Guadeloupe), Paris (France) and New Caledonia who had been previously treated for years with a single drug (some patients had been treated successively by dapsone alone and after a first relapse by rifampicin alone) yielded in 35 cases strains fully resistant to dapsone and in 13 cases strains resistant to rifampicin. Although these strains came from places where an intensive use of antileprosy drugs took place and were not representative worldwide, they demonstrate, with numerous other studies,[24,25] the reality of the drug-resistance threat. Other data which could be important for the future of leprosy control are the incidence of primary dapsone resistance. Among the 70 strains of *M. leprae* isolated from untreated patients 47 were resistant to dapsone, the majority of them of low level dapsone resistance. Although such a low level dapsone resistance is of limited clinical significance it may be considered as a first step towards a higher and more significant dapsone resistance.

Table 2. Drug resistance of *M. leprae* isolated in Paris from 1980 to 84

Strains of *M. leprae*		Susceptibility to dapsone resistant* to				Susceptibility to rifampicin†	
isolated from	N°	S	0·0001	0·001	0·01	S	R
Previously treated patients	69	7	6	21	35	56	13
Untreated patients	70	23	34	7	6	70	0

* % dapsone in the diet
† 10 mg/kg once a week

As far as we know at present one might and one should rely upon the combination dapsone–clofazimine to get rid of the RMP-resistant mutants. Then one should prescribe this combination as long as all rifampicin-resistant mutants have not been eliminated. But we do not know exactly how long the treatment with the combination dapsone–clofazimine should last to kill the 2-log population of rifampicin resistant mutants. We do know from numerous studies[17,18] that AFB from previously untreated patients who are treated with dapsone or clofazimine alone fail to grow in the normal mouse after 3–6 months of treatment and that the addition of clofazimine to dapsone increases the effectiveness of treatment. If we are optimistic we might expect the same thing to occur in patients treated with the combination rifampicin, dapsone and clofazimine, and the initial phase of chemotherapy to be capable of eliminating all drug-resistant mutants within 6 months. However if we are pessimistic we might expect rifampicin to induce a generalized state of persistence among all viable organisms that have not been killed by the initial dose of the drug. If the organisms are rendered unresponsive to rifampicin because of their metabolic inactivity they should be similarly unresponsive to the other drugs. Thus the rifampicin-resistant mutants are likely to respond badly to the combination dapsone–clofazimine. In this event the combination dapsone–clofazimine would take longer than 6 months and perhaps the whole course of chemotherapy to get rid of the RMP resistant mutants. It is, therefore, a priority to determine with precision the length of time required by the combination dapsone–clofazimine to obtain a 2–3 log decrease in the RMP-resistant population. This period should thus be the length of time of the initial phase of chemotherapy. As long as we do not have this information, and in order to remain on the safe side, it is highly recommended to give the combination dapsone–clofazimine during the whole course of chemotherapy.

3 The secondary phase of chemotherapy

The initial phase of chemotherapy having eliminated all drug-resistant mutants and reduced the size of the viable drug-sensitive population to 4 log bacilli, the secondary phase is responsible for the elimination of the remaining bacilli. But in the Bamako–Chingleput study to which reference was made above,[19] the proportion of AFB capable of multiplying in mice remained constant during the 2 years of treatment, indicating that chemotherapy was, after the initial rapid killing of *M. leprae* apparently ineffective against the remaining *M. leprae*. These *M. leprae* were not drug-resistant organisms selected by the chemotherapy because the few bacilli that grew in the footpad of mice were normally sensitive to rifampicin and other drugs. They behaved as true persisters, that is organisms unresponsive to the drugs to which they are fully susceptible.[26,27] Such persisters have also been observed in leprosy after long-term dapsone therapy and in many other infectious diseases including tuberculosis.[28–30]

Faced with the apparent unresponsiveness of *M. leprae* persisters to chemotherapy, the obvious question is whether or not we should try to get rid of the 4-log persisters because they are potentially responsible for relapses after stopping treatment. In other words should chemotherapy be continued or stopped after all drug-resistant mutants have been killed? At a time when we do not know how long it takes to kill all drug-resistant mutants, we are still more ignorant about the optimal length of the continuation phase of chemotherapy. However one tentative answer to the question of the length of the continuation phase can be brought by the results of the Bamako–Chingleput study. In this study, the load of AFB in the serial skin smears decreased by 0·62 log per year. Because the proportion of persisters among AFB remained constant (-7 log) during the 2 years of the study, it is possible to infer that the clearing of persisters was parallel to the clearing of the AFB in the skin smears and was also of 0·62 log per year. If that is right it is possible to calculate the length of time needed to clear 4-log persisters. This length of time is 4 divided by 0·62, that is 6·45 years or more simply 7 years for multibacillary cases of leprosy with maximum bacterial load, that is more or less the time needed to reach skin smear negativity. For multibacillary cases of leprosy with lower bacterial load, the length of chemotherapy would depend upon the initial load of AFB and the speed of its decrease under chemotherapy. For the previously treated patients with already negative or poorly positive skin smears, the optimal length of combined chemotherapy could well be still shorter.

To validate this reasoning, it is interesting to compare the calculated length of time needed for the chemotherapy of leprosy with rifampicin to get rid of persisters with the known length of time needed for the short-course chemotherapy of tuberculosis to be fully effective (to prevent relapses in almost 100% of the cases). Four-drug chemotherapy of tuberculosis with isoniazid, rifampicin, pyrazinamide and streptomycin (or ethambutol) should last 6 months to be followed by an acceptable relapse rate. Let us consider that the mean division time is about one day for *M. tuberculosis* (actually 20 hr) and about 14 days for *M. leprae* (actually between 12 and 20 days), i.e. 14 times longer. If the clearing of persisters under chemotherapy is related to the division time of the mycobacterial species, then one may calculate from the time needed to eliminate persisters under short-course chemotherapy of tuberculosis that the length of time needed to eliminate persisters under multidrug chemotherapy of leprosy is 6 months $\times$ 14 = 84 months or 7 years! This figure fits surprisingly well with the figure estimated from the Bamako–Chingleput study.

Finally it should be emphasized that the preceding reasoning is purely speculative and perhaps far too pessimistic. Because standard regimens of combined chemotherapy have been introduced very recently, nobody knows exactly the importance of the threat represented by the *M. leprae* that persist despite combined chemotherapy with rifampicin or even whether these persisters are actually threatening the future of multibacillary patients after stopping

treatment. Only carefully-designed long-term studies in the next 10 years will provide answers to the questions concerning the optimal length of chemotherapy for leprosy.

4 Future research in chemotherapy of leprosy

In the last decade tremendous advances were made in the chemotherapy of leprosy. At present it is generally agreed that: (i) rifampicin should as far as possible be a component of all antileprosy regimens; (ii) rifampicin should be combined with at least one and preferably two active drugs to prevent the selection of rifampicin-resistant mutants; and (iii) due to the high killing activity of rifampicin, the treatment of even the most severe lepromatous cases of leprosy does not need to be lifelong. Although there is ample indirect evidence that multidrug therapy of leprosy with rifampicin is necessary and will improve to a large extent the results of chemotherapy for leprosy control programmes, it should be realized that direct evidence will be obtained only in the range of the next 10 years. An obvious reason for this delay is the long division time of *M. leprae* (about 14 days) which seems also to be responsible for the slow evolution of the disease and for the slow motion of all events in leprosy. Another reason which is not always well understood is the limited precision of the tools (skin smear, mouse inoculation, phenolic glycolipid serology) available to assess success and failures in the chemotherapy of leprosy. Improvement in the sensitivity of the available tools and discovery of new tools to assess the response of patients to chemotherapy are therefore needed.

A number of questions, that deserve perhaps to be thought about, may be raised about the response of *M. leprae* to chemotherapy. For example one can wonder whether the rapid initial killing of *M. leprae* by rifampicin is due to the poor viability of the microbial cells at the beginning of treatment or to the special susceptibility of the target of rifampicin, the RNA polymerase of *M. leprae*. It is striking that one single dose of 1500 mg rifampicin combined with daily dapsone is capable of killing 5 log *M. leprae* in a few days whereas it takes at least 2 months of daily treatment with the combination of three bactericidal drugs, namely rifampicin, isoniazid and pyrazinamide, to kill 5 log *M. tuberculosis*.[5] If the *M. leprae* RNA polymerase was actually different from that of *M. tuberculosis* and other bacteria[31] that would explain why the effectiveness of rifampicin against all of the bacterial species except *M. leprae* decreases when the interval between each individual 10 mg/kg dose increases.[32]

Another striking finding of the Bamako–Chingleput study is the apparent unresponsiveness of *M. leprae* to rifampicin during treatment after the initial dramatic response. This secondary unresponsiveness might well be the result of some special interaction between the host macrophage and *M. leprae* or of the chemical structure of *M. leprae* RNA polymerase or both. If the first hypothesis

was right, then it should perhaps be possible to improve the response of *M. leprae* persisters to rifampicin by combining immunotherapy and chemotherapy. Such an hypothesis is certainly worth testing experimentally and clinically. If the second hypothesis was right, then the identification of the *M. leprae* RNA polymerase could be of utmost importance to understand the mechanisms of action of rifampicin against *M. leprae* and to improve the effectiveness of multidrug therapy.

Among other directions for future research, the relationship between the presence of persisters and the risk of relapse after stopping treatment is certainly one that deserves high priority. *M. leprae* persisters have been isolated after a long course of dapsone monotherapy[30] as they have been isolated after a long course of multidrug therapy.[26,27,33] But we do not know whether the size of the persisters population is similar after dapsone monotherapy and after multidrug therapy. Similarly, we do not know whether there is a relation between the size of the persisters population, the risk of relapse and the length of time that elapses between the end of chemotherapy and the relapse. To answer these questions experimental and clinical investigations are certainly needed. But it should be recognized that they will be difficult and costly to perform.

It should be recalled that at present only four drugs, namely rifampicin, the thionamides, dapsone and clofazimine, are active against *M. leprae* and only rifampicin has a strong bactericidal activity. Therefore chemotherapy of leprosy needs new compounds with a bactericidal activity to back up rifampicin and to overcome the increasing prevalence of dapsone resistance.[25] Among new compounds active against *M. leprae* only the new fluoroquinolones appear promising.[34] Their activities in the mouse and even in man are still under study but the initial results are favourable. Finally one can hope that fundamental research involving all new tools used in molecular biology will permit us not only an understanding of the mechanisms of action of the already known antileprosy drugs, and to improve their use, but also to discover new leads.

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Strategies in the development of new drugs and drug combinations against leprosy, demonstrated on the example of folate and gyrase inhibitors

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According to the reports of the pharmaceutical industry 5000–10,000 compounds have to be synthesized to get one compound introduced into therapy.

The aim of the modern approaches in drug design which can be summarized under the title 'Quantitative Structure–Activity Relationship (QSAR)' analysis is to change this uneconomic ratio, i.e. to reduce the number of derivatives which have to be synthesized, to decrease costs but also the number of experimental animal studies. This is especially important for the development of antileprotic drugs where drug development is mainly performed in research laboratories outside the pharmaceutical industry.

It is without doubt that the further development of suitable *in vitro* test systems, increased knowledge in molecular biology and the application of modern computerized techniques for the analysis of quantitative structure–activity relationships can serve to reach this aim.

The position of the modern medicinal chemist in comparison to his predecessor, who had to face these problems 20 years ago, is much more favourable. The optimization of drugs is much less dependent on chance and chance correlation. The reasons for this are better information about biological systems (metabolic pathways, enzyme kinetics, X-ray structure of receptors etc.), the meaning of structural molecular properties, their manipulation and the knowledge of the importance of various computerized methods for data analysis.

With these methods, which will be discussed in a relation to a few selected examples, it is possible to speed up the optimization of lead compounds within a congeneric set of derivatives, to detect and quantify outliers and to gain information on the mechanism of action.[1]

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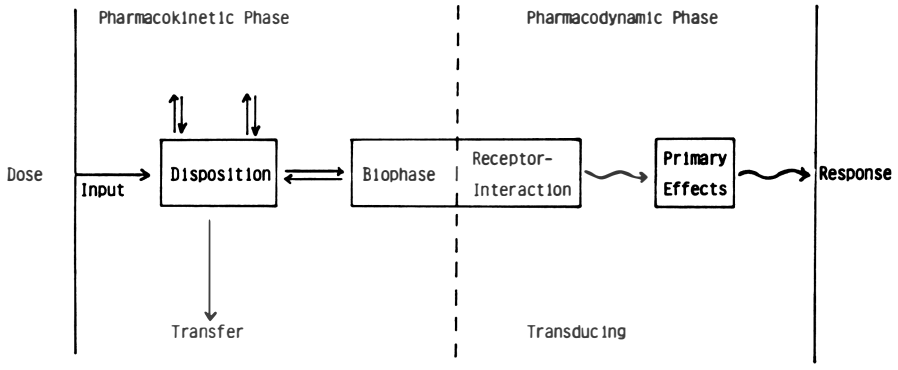


Figure 1. Schematic drawing of steps involved in drug action.[10]

The major problem in the application of these techniques is the complexity of the biological system and of the steps involved in drug action.

This is demonstrated in a simplified drawing in Figure 1. A dose of a drug is administered to a biological system, for example an experimental animal. The dose gets absorbed to a certain extent, is distributed, metabolized, bound to serum and tissue constituents and eliminated. These complex pharmacokinetic events determine the amount of drug which reaches the target in the biophase. A response is released which is measured as a pharmacodynamic effect. Even if we could succeed in relating the degree of the measured effect released by various derivatives to certain molecular properties, it is almost impossible to deduce the causal connection because of the complex nature of the system. In case of the development of antibacterial drugs we are in a somewhat better position. We can measure the inhibitory effect outside the host organism. But even then several steps can be rate limiting, i.e. become decisive for no, some or excellent effect. This is demonstrated in Figure 2. The rate limiting step can be the permeability of the bacterial cell wall, the binding of the drug to the target enzyme or its reactivity to form products, toxic to the bacterial cell.

In leprosy we have the special and unfortunate situation that *Mycobacterium leprae* does not multiply under *in vitro* conditions, i.e. in a test tube. So far for the determination of inhibitory effects of compounds only the mouse footpad technique has been used with the limitations just discussed. We have also to realize that so far neither new drugs have been found nor existing drugs have been developed by this technique. All antibacterials used have been developed against other infections, especially mycobacteriosis including tuberculosis. These drugs dapsone, rifampicin, clofazimine, prothionamide—to name some of them—are all active against a variety of mycobacterial strains; the ranking in activity is sometimes changed comparing results from different bacterial strains. As long as

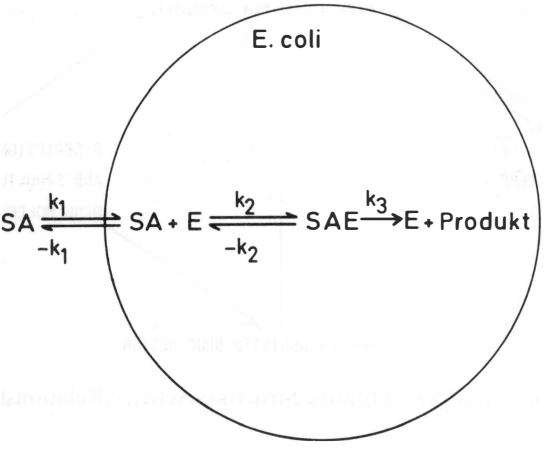


Figure 2. Possible rate limiting steps in antibacterial action.

no breakthrough in cultivation of *M. leprae* or in the transformation of genetic information from *M. leprae* to a cultivable bacterium is achieved the following procedures can be helpful for a systematic optimization of known drugs and also to generate new leads for therapeutics against leprosy:

1 Use of model mycobacterial strains as *in vitro* screening system.
2 Use of isolated target enzymes derived from mycobacteria, including armadillo derived *M. leprae*, for optimization of activity and to detect possible problems in cell-wall permeability.
3 Study of pharmacokinetics and serum activity tests in healthy volunteers after completion of toxicity studies in experimental animals.

The information about the inhibitory activity obtained in these test systems is used to find those structural properties of the drug molecule, which are decisive for the inhibitory power *in vitro*. Various techniques of structure–activity relationship analysis are applied to detect such correlations. A general outline of the procedure is given in Figure 3.

A variety of descriptors can be used to describe certain structural or physicochemical properties of the drug molecules. Essential are often changes in lipophilic, electronic or steric properties. For detailed information one has to refer to the literature.[1–3]

Especially for the chemotherapy of leprosy another aspect is important in drug development, this is the combination of drugs. Again systems have to be developed which allow the quantification of possible synergistic or antagonistic effects of drug combination. Today it is generally agreed that multidrug therapy is a must in chemotherapy of leprosy.[4–8]

And last not least the inhibitory power of a certain derivative is not the only

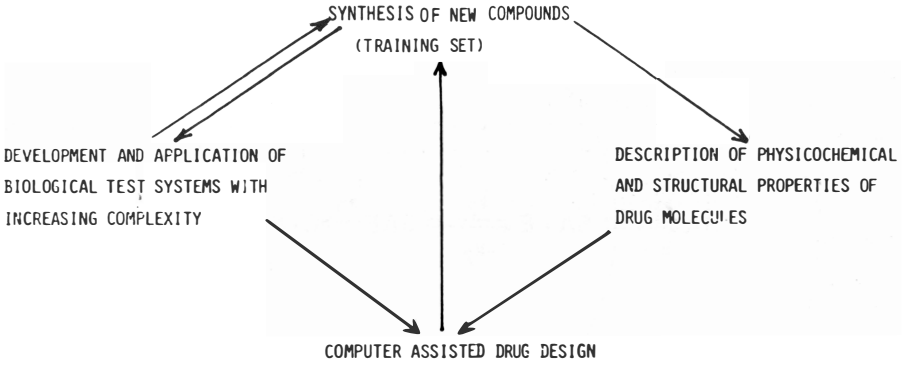


Figure 3. Diagram of steps in Quantitative Structure-Activity Relationship (QSAR) analysis.

parameter important for a compound to become a useful drug. Some other factors which have to be considered in the optimization procedure are listed in Table 1.

For the last points mentioned in this table, the analysis of the structural dependence of the pharmacokinetic properties is of the utmost importance.[9,10]

This general strategy is outlined on the example of folate inhibitors. The folate synthesizing enzyme system is an excellent target for drugs. The folate pathway is given in Figure 4. Dapsone (DDS) is a well known and powerful inhibitor of the pteroic acid synthetase of *M. leprae* and *M. lufu*. The question is if a further increase in activity can be achieved or if pharmacokinetic properties or tolerance can be optimized. The second suitable target in this enzymatic pathway is the dihydrofolate reductase (DHFR). A known powerful inhibitor is trimethoprim which is, however, restricted to gram negative bacteria only. The question is: is it possible to design a trimethoprim derivative with inhibitory activity against DHFR of mycobacteria. A combination of such a derivative with dapsone or a dapsone derivative could be of great interest for the therapy of leprosy.

Table 1. Possible aims in drug optimization.

1	Extension of range of activity
2	Increase in biological activity
3	Increase in specificity (decrease in toxicity)
4	Change in pharmacokinetic properties: bioavailability; biological half-life; metabolism; distribution etc
5	Adaption of pharmacokinetics to other drugs used in combinations

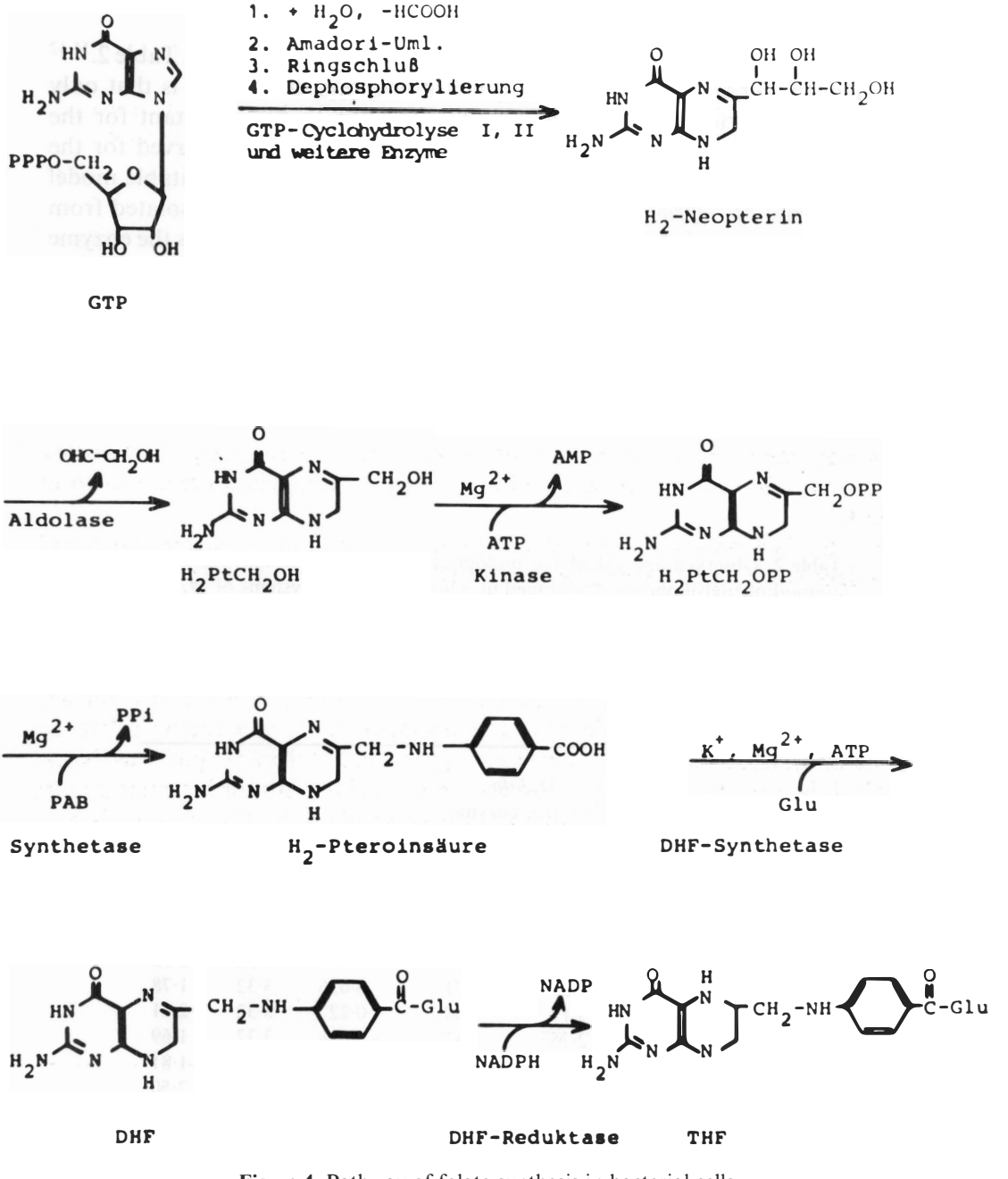


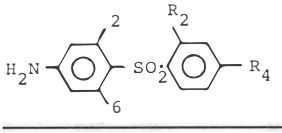
Figure 4. Pathway of folate synthesis in bacterial cells.

Inhibitors of Dihydropteroic Acid Synthetase

The results of our optimization efforts for dapsone are summarized in Table 2.[11,12] The achieved increase in activity (eq 1) is not exciting. Of interest is that only electronic and steric effects of the substituents seem to be important for the observed differences in activity and that the same ranking is observed for the cultivable *M. lufu* and for *M. leprae* indicating that *M. lufu* is a suitable model strain to mimic *M. leprae* in this respect. In addition the enzyme isolated from both resistant strains shows the same sensitivity towards sulphones as the enzyme derived from sensitive strains (Table 3). This allows the conclusion that the resistance is not due to changes in the structure of the target enzyme.

Changes in the lipophilic properties of the derivatives are not responsible for the observed differences in their activity in cell-free systems. Therefore it is possible to change the lipophilicity without affecting the antibacterial activity

Table 2. Observed and calculated biological activity data of substituted 4′-aminodiphenylsulphones determined in cell-free (I_{50}, μM) systems of *M. lufu* and physicochemical descriptors used in QSAR analysis.



R_2	R_4	*M. lufu*$_s$ cell-free enzyme I_{50} [μM] obs./calc. (eq.1)		Δppm 2/6H	V_{w2}	log k_r'
H	NH_2(DDS)	1·20	1·59	−0·108	3·32	0·488
H	$NHCOCH_3$	4·05	4·20	−0·036	3·32	1·003
H	Br	9·69	6·83	0·00	3·32	2·38
H	$COOCH_3$	11·69	9·71	0·026	3·32	1·78
H	COOH	3·17	3·52	0·22	3·32	−2·11
H	NHC_2H_5	2·75	1·82	−0·098	3·32	1·69
H	$N(CH_3)_2$	2·39	2·05	−0·089	3·22	1·81
OCH_3	NHC_2H_5	1·72	1·24	−0·114	16·07	2·50
Cl	$NHCH(CH_3)_2$	0·86	1·17	−0·106	12·00	2·75
CH_3	NH_2	0·65	0·46	−0·178	13·67	0·94
CH_3	NHC_2H_5	1·09	0·47	−0·177	13·67	2·11

$\log 1/I_{50} = -5{\cdot}85\Delta\text{ppm } 2/6H + 0{\cdot}066\ V_{w2} - 0{\cdot}0031\ V_{w2}^2 + 0{\cdot}42\ f_{ion.} - 1{\cdot}06$ (eq. 1) $n = 50\ r = 0{\cdot}876\ s = 0{\cdot}21\ F = 37$.

Table 3. Comparison of biological activities of 4-substituted 4-aminodiphenylsulphones determined in cell-free enzyme systems of DDS-sensitive and DDS-resistant strains of *M. lufu* and *M. leprae* and QSAR-equations derived.

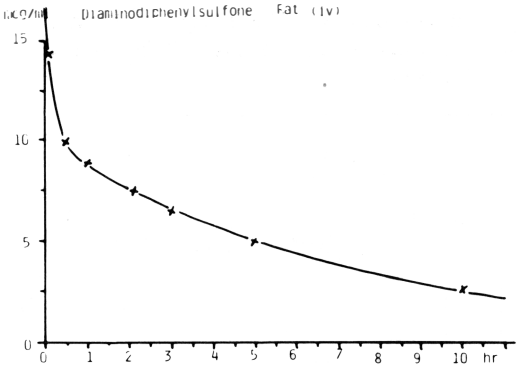
		i_{50} (cell-free enzyme system) μM				
R_2	R_4	*M. lufu*$_s$	*M. lufu*$_r$	*M. leprae*$_s$	*M. leprae*$_r$	*E. coli*
H	NH_2 (DDS)	1·20	1·18	0·42	0·28	35·3
H	NHC_2H_5	2·75	1·50	1·16	0·98	41·4
H	$NHCOCH_3$	7·0	4·05	1·88	1·26	81·6
H	OCH_3	6·0	9	—	2·87	115
H	$CONHNH_2$	12·7	9·6	4·58	3·45	164·3
H	Cl	13·0	18·7	7·16	5·33	—
H	NO_2	31	104	31·00	28·7	221

	n	r	s	F
eq. 2: $\log(1/i_{50\,M.\,leprae})^s = -7{\cdot}88(2{\cdot}56)\Delta ppm2/6H - 0{\cdot}739(0{\cdot}24)$	8	0·95	0·22	56·7
eq. 3: $\log(1/i_{50\,M.\,lufu})^s = -7{\cdot}92(1{\cdot}62)\Delta ppm2/6H - 1{\cdot}03(0{\cdot}10)$	14	0·95	0·15	112·0
eq. 4: $\log(1/i_{50\,M.\,leprae})^r = -9{\cdot}59(4{\cdot}5)\Delta ppm2/6H - 0{\cdot}65(0{\cdot}28)$	7	0·925	0·26	29·9
eq. 5: $\log(1/i_{50\,M.\,lufu})^r = -9{\cdot}21(2{\cdot}36\Delta ppm2/6H - 1{\cdot}15(0{\cdot}15)$	14	0·926	0·22	72·6

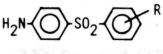
significantly. By these changes, however, pharmacokinetic properties can be altered. The result for a few derivatives is shown in Figure 5.[13] The increase in lipophilic properties of the drug decreases the clearance, i.e. increases the biological half-life of the compounds. This is important to adopt the pharmacokinetics of dapsone derivatives to the pharmacokinetics of other drugs which can be used in combination. A 'side effect' of this development was the decrease in toxicity. Some of the highly active new dapsone derivatives do not show the formation of methemoglobin in the cat even at high concentrations (Table 4).[14]

DHFR inhibitors

The second example of a 'rational' drug design is the development of a new DHFR inhibitor. Starting point was the observation that trimethoprim (TMP) shows no significant inhibitory activity against mycobacteria, however, to our surprise relatively strong inhibitory activity against the isolated target enzyme (Table 5). This seems to indicate limited permeability of the drug into the bacterial cell. The second consideration came from the knowledge of the X-ray structure of the DHFR.[15,16] It shows a positively charged arginine moiety in the cavity of the active centre of the enzyme. Other groups[16] had already shown that



Dependence of renal clearance of sulfones on their lipophilicity (rats, i.v. administration)



	log k_r'	Cl_{renal} [ml/min]
4-NH_2	0.488	0.919
4-OH	-1.067	3.35
4-COOH	-2.11	3.31
4-$NHCH_3$	1.288	0.789
18	0.09	1.96
19	1.339	0.72

$\log Cl_{tot} = -0.21(\pm 0.03)\log k_r' + 0.18(\pm 0.04)$

$n = 6 \quad r = 0.93 \quad s = 0.12$

Figure 5. Dependence of renal clearance (Cl_{renal}) of sulphones on their lipophilicity expressed as capacity factor, log *k,'* determined by HPLC-analysis (rats, i.v. administration).

Table 4. Observed methemoglobin formation in cats after p.c. administration of 200 mg/kg of the indicated sulphones.[14]

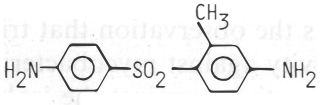
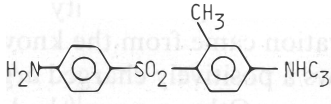
	5 hr	24 hr
Dapsone	6·09 %	4·4 %
	0·7 %	1·2 %
	0 %	0 %

Table 5. Observed biological activities of marketed and newly synthesized dihydrofolate reductase inhibitors against cell-free and whole cell systems of various mycobacteria.

	M. lufu I_{50}[μM]		MIC [μM]			
Compound	growth kinetics	cell-free enzyme*	*M. lufu*	*M. tub.* H37Rv	*M. marin.* SN1254	*E. coli*
Trimethoprim	95	0·07	> 110	> 110	28	1·4
Brodimoprim	45	0·06	80	94	18	1·4
Tetroxoprim	213	0·33	—	71	—	11·25
Diaveridin	—	—	> 123	> 123	46	8
Pyrimethamin	—	0·47	210	129	—	—
GH305	—	0·16	25·3	51	51	22·5
GH306	—	0·17	—	97	48	—
GH307	—	0·15	23	70	46	> 45
GH308	—	0·81	22·3	33	5·6	22·5
GH310	—	1·11	31	10	2·6	4·0
K107	1·37	0·06	2·3	29	3·6	> 45
K120	—	—	32·0	65	33	> 90
K122	—	1·58	> 58	3·4	13	11·25
K128	8·31	0·03	12·2	65	6·1	> 45
K130	1·66	0·01	0·7	5·3	7·1	32
K132	—	0·26	11·0	7·3	16	11
K135	—	—	—	7·1	14	11
K137	—	0·04	1·9	2·8	30	> 90
K138	—	0·08	3·8	7·7	> 90	—

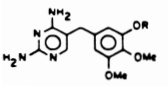
* 10 μM DHF

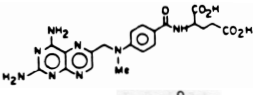
TMP derivatives bearing as substituents negatively charged ionized carboxy groups, led to an increased activity against the isolated enzyme (Figure 6); no activity, however, against the whole cells; this indicates problems in the permeation of the bacterial cell wall. We have therefore systematically synthesized a series of derivatives which possess higher lipophilicity and in addition a negatively charged but unionized group. The results are summarized in Table 5. The high inhibitory power—activity is increased by a factor of more than 100—is also shown by the bacterial growth kinetic experiment using *M. lufu* as model strain (Figure 7).

The results obtained[17,18] in *M. leprae* suspensions are encouraging where the ATP-level and ^{3}H-thymidine uptake is determined in the absence and presence of these derivatives. A strong inhibitory effect is observed (Table 6). An inhibitory

TMP analogs as inhibitors of E.coli DHFR

L.F.Kuyper et al., J.Med.Chem. 25,1120(1982)





R	$K_i \cdot 10^9$
$-CH_3$ (trimethoprim)	1.3
$-CH_2COOH$	2.6
$-(CH_2)_2COOH$	0.37
$-(CH_2)_3COOH$	0.035
$-(CH_2)_4COOH$	0.066
$-(CH_2)_5COOH$	0.024
$-(CH_2)_6COOH$	0.050

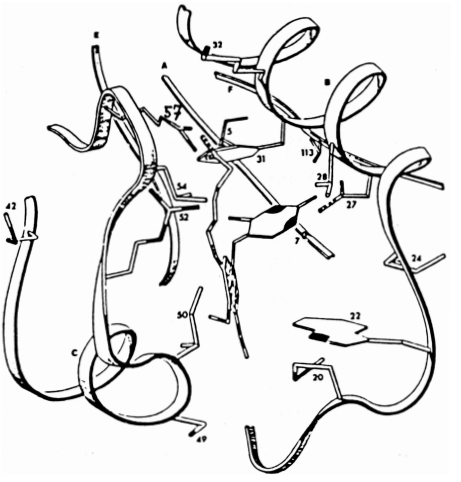


Figure 6. X-ray crystallography of *E. coli* derived DHFR and of its binary complex with a DHFR inhibitor (trimethoprim).[15]

effect can also be demonstrated in mice. The inhibitory effect is delayed despite the fact that the achieved blood levels of 3–4 μg/ml are well above the MIC of the derivative K-130 (Table 7). This is, however, not surprising because it is well documented that low inhibitory activities of DHFR inhibitors, as for example TMP, are found in mice despite the fact that this drug is an excellent chemotherapeutic in human infectious diseases caused by Gram negative bacteria.

This is leading back to the starting point of this paper. It seems not justified to rely only on mouse footpad experiments in developing new drugs, it may lead to unjustified decisions, the risk of producing false negatives cannot be excluded; K-130 seems to be a good example.

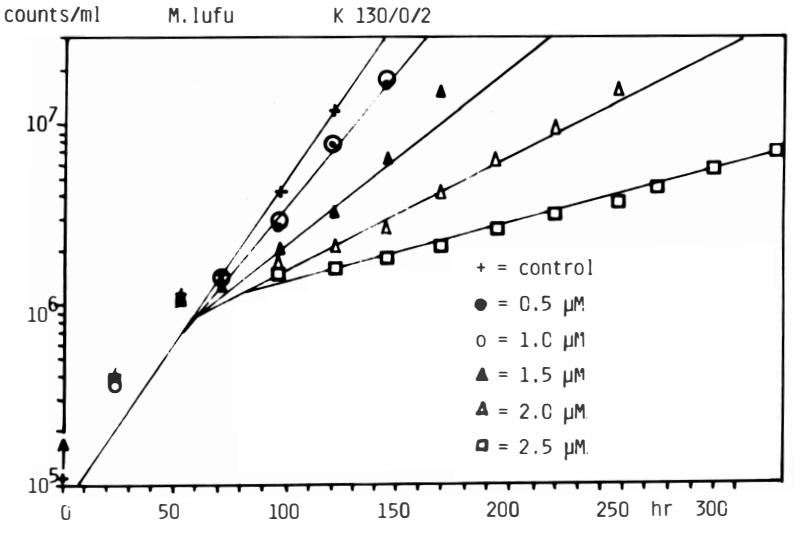


Figure 7. Typical generation rate curves of *M. lufu* at 30°C in the presence of concentrations of K-130 as indicated. +, control; ●, 0·5 μM; ○, 1·0 μM; ▲, 1·5 μM; △, 2·0 μM; □, 2·5 μM.

Table 6. Inhibitory effect on ^{3}H-thymidine uptake and ATP-level on *M. leprae* suspensions.[17]

Drug (DDS derivative)			Percent inhibition of *M. leprae* multiplication at drug concentrations (ng/ml)								
R_2	R_4		0	5	10	15	20	25	30	40	50
H	NH_2	a	0	0	0	15	80	100	100	100	100
		b	0	0	0	13	79	100	100	100	100
H	NHC_2H_5	a	0	0	0	0	0	0	0	17	17
		b	0	0	0	0	0	0	0	17	19
H	Cl	a	0	0	0	0	0	0	0	0	0
		b	0	0	0	0	0	0	0	0	0
128-0-2-KA		a	0	0	0	0	18	21	48	48	47
		b	0	0	0	0	17	22	48	50	45
130-0-1		a	0	0	0	0	61	82	100	100	100
		b	0	0	0	0	59	79	100	100	100

a = ATP assays; b = [^{3}H] Thymidine uptake assay

Table 7. Footpad harvest of *M. leprae* infected mice after the indicated length of treatment with K 130.[18]

	$\times 10^6$ *M. leprae*/foot pad			
	4 months	6 months	7 months	9 months
control (%)	0·826±0·089	1·69±0·10	2·47±0·169	3·41±0·113
DDS 0·0001	0·78±0·083	1·45±0·10	1·85±0·193	2·35±0·08
K-130 0·03	0·47±0·063	0·41±0·043	0·13±0·025	0·0
K-130 0·03 + DDS 0·0001	0·40±0·069	0·30±0·095	0·07±0·023	0·0

Development of drug combinations

The last aspect to be discussed is the analysis of the effects of drug combinations and how to quantify possible synergistic, additive or antagonistic effects of drug combinations. There is no doubt that it could be very dangerous to combine drugs only on the basis of the activity observed for the single drug.

Suitable 'test systems' for such an analysis are the bacterial growth kinetic[19,20] and the checkerboard technique.[21] An example for the strong synergistic action of the DHFR blocker, Brodimoprim® or the new derivative K-130, respectively, with dapsone is shown in Figure 8(a) and (b).

Identical results are obtained by the checkerboard technique (Table 8). Table 9(a) and (b) shows an example of a combination where the combined action is less compared to the inhibitory activity of the single drugs. Such a combination cannot be recommended. Synergistic and antagonistic effects are mechanistically based and will be similar for various types of bacteria showing sensitivity against the single drugs.

Gyrase inhibitors

Another class of compounds which seems to be of interest for the chemotherapy of leprosy are the newly developed quinolones. These compounds are supposed to inhibit the enzyme gyrase responsible for the coiling of DNA. Several derivatives have been synthesized by various pharmaceutical companies and have been tested against various bacterial strains. So far the most effective derivative against the majority of the sensitive strains seems to be ciprofloxacin. It has a broad antibacterial spectrum including mycobacteria (Table 10). The results of a growth kinetic experiment with *M. lufu* are shown in Figure 9.

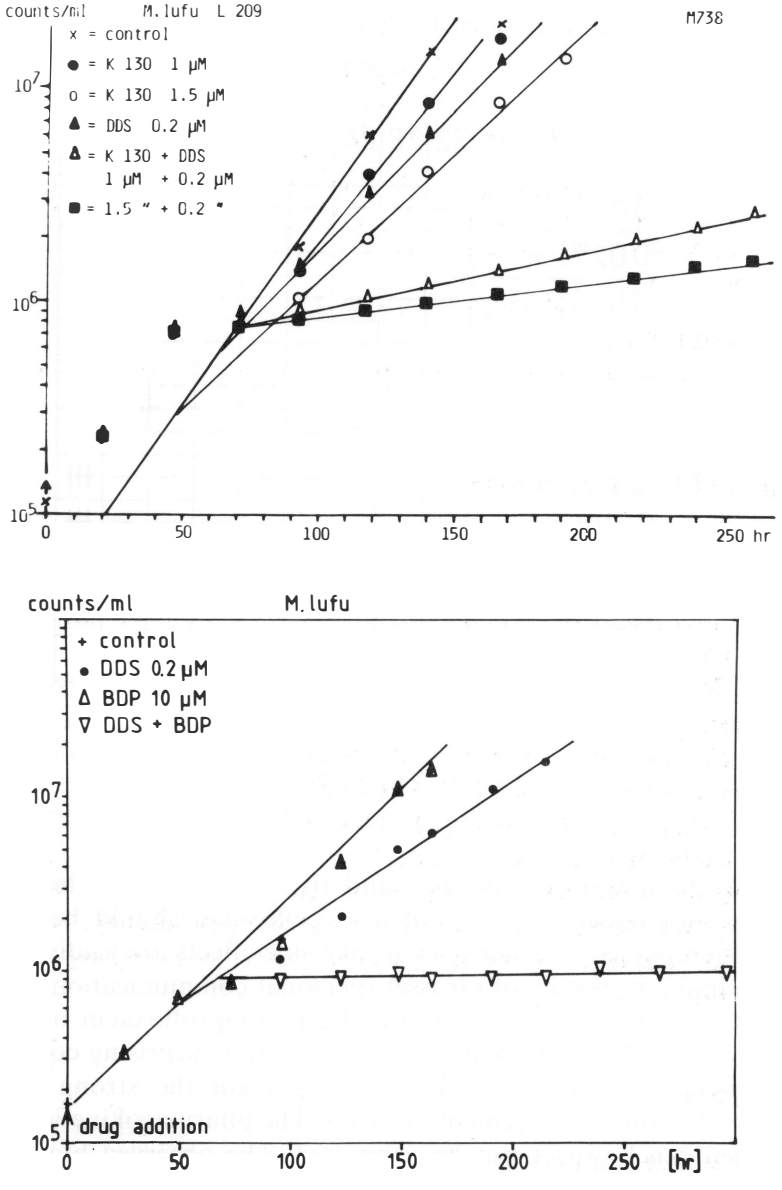


Figure 8. Bacterial generation rate of *M. lufu* at 31°C in the presence of (a) K 130 and dapsone (DDS) alone and in combination, x, control, ●, K130 1μM; ○, K130 1·5 μM; ▲, DDS 0·2 μM; △, K130+DDS 1μM+0·2 μM; ■, 1·5′ μM+0·2′ μM; and (b) brodimoprim (BDP) and dapsone (DDS) alone and in combination at the concentrations indicated. +, control; ●, DDS 0·2 μM; △, BDP 10 μM; ▽, DDS+BDP.

Table 8. Antibacterial effects of brodimoprim (BDP) and dapsone (DDS) alone and in combinations on *M. lufu*. − = growth; + = no growth. Fractional inhibition index (FII) calculated. Strain, *M. lufu*; medium, Dubos + albumin; inoculum, 5×10^{-5} mg germs.

DDS \ Brodimoprim	40	36	32	28	24	20	16	12	8	4	0
						mcg/ml					
0,03	−	−	−	−	−	−	−	−	−	−	−
0,027	−	−	−	−	−	−	−	−	−	−	+
0,024	−	−	−	−	−	−	−	−	−	−	+
0,021	−	−	−	−	−	−	−	−	−	−	++
0,018	−	−	−	−	−	−	−	−	−	−	+++
0,015	−	−	−	−	−	−	−	−	−	−	+++
0,012	−	−	−	−	−	−	−	−	−	−	+++
0,009	−	−	−	−	−	−	−	−	−	−	+++
0,006	−	−	−	−	−	−	−	−	−	(1)	+++
0,003	−	−	−	−	−	−	−	−	(2)	+	+++
0 mcg/ml	−	+	+	++	+++	+++	+++	+++	+++	+++	

F.I.I.
(1) 0,30
(2) 0,21

According to our results *in vitro* and the serum activity tests, not only ciprofloxacin and ofloxacin[17,18,24] but also pefloxacin should be suitable for treatment of leprosy, if no other reasons like side-effects are leading to another decision. Preliminary results of Grosset (personal communication) obtained in mouse footpad experiments indicate no effect of ciprofloxacin but significant antileprotic activity for pefloxacin. This result is not surprising considering the pharmacokinetic data available. It indicates again the strong influence of pharmacokinetics on the judgement of drugs. The pharmacokinetics of drugs in mice and men can however not be compared. The observed blood levels for ciprofloxacin in man should be sufficient for therapy (Figure 10), especially as ciprofloxacin shows an extremely good distribution into various tissues and also an accumulation in various cells. This observation is of great importance because mycobacteria are intracellularly growing organisms. First results on combinations of ciprofloxacin with other drugs are not conclusive. No significant synergism was observed for combinations with DDS, prothionamide or brodimoprim. The combination with rifampicin seems to tend to be slightly antagonistic.

Table 9 (a) and (b). Antibacterial effects of rifampicin (RMP) and lamprene (LP) alone and in combination on *M. lufu* and *M. marinum* showing antagonistic effects. LP, lamprene; RMP, rifampicin, (a) strain, *M. lufu*, (b) *M. marinum* SN1254.

(a)

RMP \ LP	0.05	0.045	0.04	0.035	0.03	0.025	0.02	0.015	0.01	0.005	0 µg/ml
0.05	–	–	–	–	–	–	–	–	– (4)	–	–
0.045	–	–	–	– (3)	(+)	+	+	+	+	+	+
0.04	–	–	–	+	+	+	+	+	+	+	+
0.035	–	–	–	+	+	+	+	+	+	+	+
0.03	–	–	–	+	+	+	+	+	+	+	+
0.025	–	–	–	+	+	+	+	+	+	+	+
0.02	–	–	– (2)	+	+	+	+	+	+	+	+
0.015	–	–	+	++	++	++	+++	+++	+++	+++	+++
0.01	–	– (1)	+	++	+++	+++	+++	+++	+++	+++	+++
0.005	–	–	+	++	+++	+++	+++	+++	+++	+++	+++
0 µg/ml	–	+	++	+++	+++	+++	+++	+++	+++	+++	

FII

(1) = 1.1; (2) = 1.2; (3) = 1.6; (4) = 1.2

(b)

RMP \ LP	0.4	0.36	0.32	0.28	0.24	0.20	0.16	0.12	0.08	0.04	0 µg/ml
2.0	–	–	–	–	–	–	–	–	–	–	–
1.8	–	–	–	–	–	–	–	–	–	–	–
1.6	–	–	–	–	–	–	–	–	– (3)	–	–
1.4	–	–	–	–	–	–	– (2)	(+)	+	+	+
1.2	–	–	–	–	–	+	+	++	++	++	++
1.0	–	–	–	–	–	+	+	++	++	++	++
0.8	–	–	–	–	– (1)	++	++	+++	+++	+++	+++
0.6	–	–	–	–	+	++	+++	+++	+++	+++	+++
0.4	–	–	–	–	+	++	+++	+++	+++	+++	+++
0.2	–	–	–	–	+	++	+++	+++	+++	+++	+++
0 µg/ml	–	–	–	–	+	++	+++	+++	+++	+++	

FII

(1) = 1.37; (2) = 1.44; (3) = 1.28

Table 10. Antibacterial activity of ciprofloxacin against various mycobacteria compared to some tuberculostatic drugs. (Range MIC, μg/ml)

	Medium Dubos/albumin	Medium Lockemann + 0·5 % albumin							
Strain	Ciprofloxacin	Ciprofloxacin	RMP	INH	PTH	EMB	SM	KM	CM
M. tub. H37Rv	0·125	0·25–1	0·06–0·125	0·015–0·03	1–2	4–8	2–4	2–4	2–4
M. lufu	0·06–0·2								
M. lufu DDS res.	0·06								
M. smegmatis ATCC 607	0·06–1	0·125–0·5							
M. smegmatis DDS res.		0·25–0·5							
M. marinum SN 1254	0·125	0·5–1							
M. avium SN 304	0·5–1	2							
M. avium SN 403	0·125	0·125–0·25							
M. smegmatis SN 46	0·25–0·5	0·25–1							
Patient-Strain									
M. tub. Ro.		0·25	32						
M. tub. Pa.		0·125–0·5	> 32						
M. tub. Br.		0·25–0·5	32						
M. tub. Fi.		0·25	> 32						
M. africanum Ho.		0·25	> 32						
M. shimoidei Schi.		0·5	32						
M. avium Ri.	0·5	1–2	1–4						
M. avium Ra.		16	0·5–1						

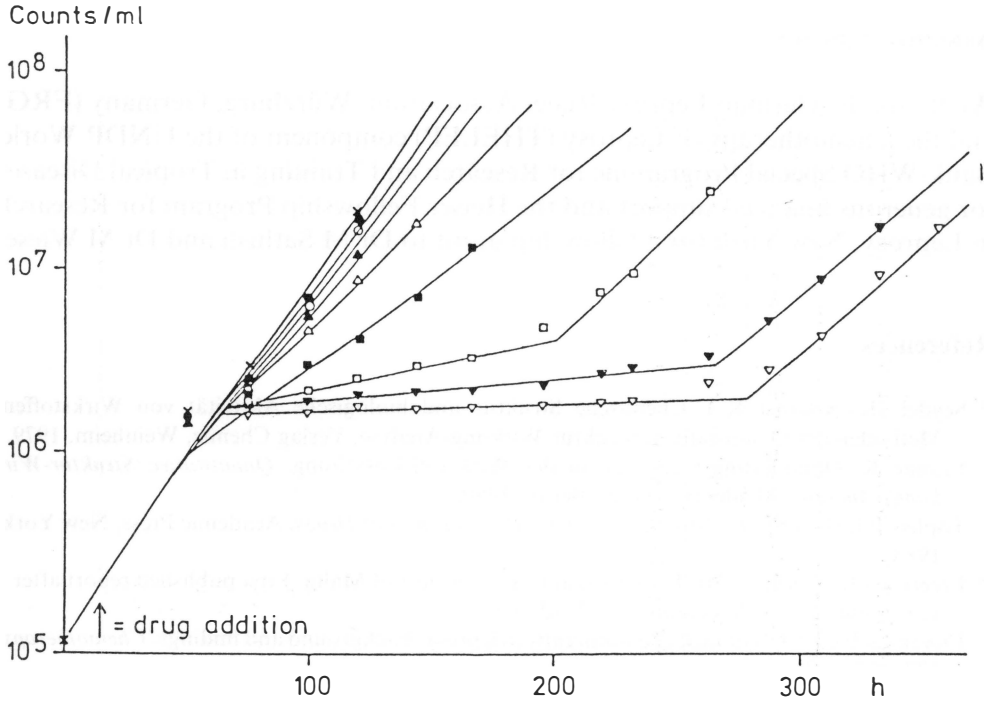


Figure 9. Typical generation rate curves of the mycobacterial strain *M. lufu* at 31°C in the presence of various concentrations of ciprofloxacin (electronic, total counts). The curves, generation rate constants k_{app} ($s^{-1} \times 10^{-5}$) and the concentrations (μM) were as follows for the first inhibited phase: x, control 1·17, 0·0; ●, 1·26, 0·06; ○, 1·18, 0·08; ▲, 1·00, 0·10; △, 0·82, 0·12; ■, 0·56, 0·14; □, 0·22, 0·16; ▼, 0·09, 0·18; ▽, 0·06, 0·20. From (24).

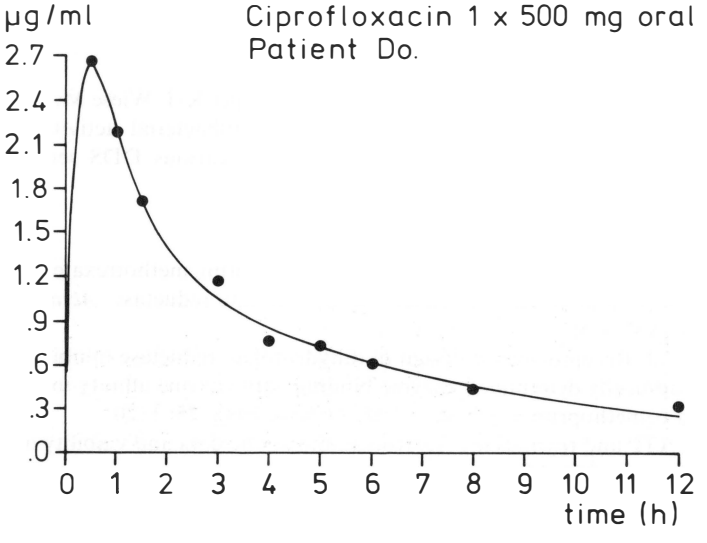


Figure 10. Plasma concentration profile (HPLC) after a single oral dose of 500 mg ciprofloxacin (patient Do.) and serum activity determination after 1 and 4 hr using bacterial strains as indicated.[24]

Acknowledgments

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Lepr Rev (1986) **57,** Supplement 3, 254–264

Development of inhibitors of mycobacterial ribonucleotide Reductase

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New drugs for the therapy of leprosy have to be developed because of the small number of existing active drugs and because of the increasing resistance of *Mycobacterium leprae* against them.

One possibility to initiate the development of a new drug is to start with a known chemotherapeutic. Our project started with the investigation of a series of analogues of thiacetazone (*p*-AcNH-Ph-CH = NNHCSNH$_2$). Unfortunately no remarkable activity against our leprosy model strain *M. lufu* and several other mycobacterial strains could be found in the class of substituted benzaldehyde thiosemicarbazones (TSCs).

Quite unremarkable activities were also found for the first two heterocyclic TSCs in Table 1. But there is a considerable increase in activity in case of α-heterocyclic TSCs as shown by the third derivative.

Similar increases in activity on going from α-nonheterocyclic TSCs to α-heterocyclic analogues have been published for the antitumor potency of these compounds.[1] Several groups have shown that the antitumor activity of these α-heterocyclic drugs is caused by the inhibition of DNA synthesis.[2–7] Their site of action is the iron-containing enzyme ribonucleoside diphosphate reductase (RDR).

RDR together with the dithiolprotein thioredoxin promotes the reduction of ribonucleotides to deoxyribonucleotides which are essential intermediates in the synthesis of DNA (Figure 1).

By Sartorelli[2,8,9] some evidence has been provided that the non heme-iron atom in the active site of the reductase interacts with α-heterocyclic TSCs which are known to be tridentate chelators.

As it has been found that bacterial RDRs show some similarities to the mammalian enzyme[1,2,10–12] we suppose that the TSCs have the same mode of action in the antitumor test and in our antibacterial test system.

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Table 1. Minimum inhibitory concentrations (MIC) of pyridine-aldehyde-thiosemicarbazones towards *M. lufu*.

R-CH = N-NH-CSNH$_2$	MIC [μM/l]
R = 4-pyridyl	611
3-pyridyl	556
2-pyridyl	40

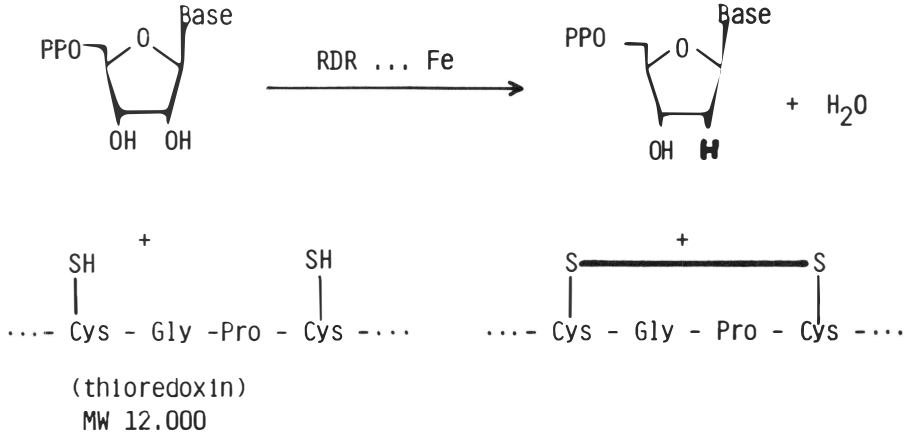

Figure 1. Reduction of ribonucleoside diphosphates by thioredoxin catalysed by the iron-containing enzyme ribonucleoside diphosphate reductase (RDR).

To investigate this hypothesis we tested a series of pyridine-2-aldehyde-TSCs and found that derivatives with substituents in the 6-position of the pyridine ring have low activities. As steric effects should be unfavourable for the formation of iron-complexes this result seems to support our hypothesis.

Several of our derivatives have also been tested by French and coworkers[1] for their inhibitory activity against RDR from human sarcoma cells.

A plot of the antimycobacterial potency of these compounds against their antitumor activity shows some colinearity (Figure 2) and therefore again seems to support the hypothesis of ribonucleotide reductase inhibition in *M. lufu*.

Pyridine-2-aldehyde-TSC derivatives are clearly more active against mycobacteria than thiacetazone, but unfortunately they are also clearly more toxic towards mammals (Table 3). The reason probably is increased delivery of toxic hydrogen sulphide.

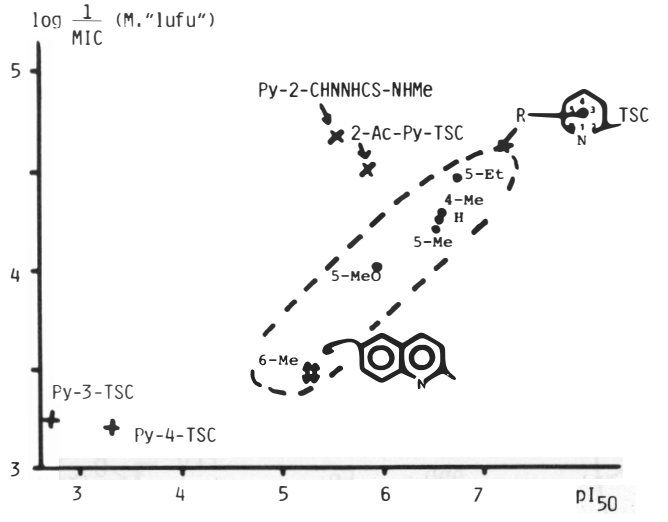

Figure 2. Plot of the antimycobacterial potency (log *1*/MIC, *M. lufu*) of acylpyridine-thiosemicarbazones *vs.* their antitumor activity (pI_{50}, in vitro inhibition of RDR from human epidermoid carcinoma).[1]

For this reason we started the investigation of a series of heterocyclic hydrazones which are not TSCs and cannot split off hydrogen sulphide. The general structure of these compounds is compared in Figure 3 with the previously discussed TSCs. For patent reasons the exact structure cannot be given.

Some antimycobacterial test results of two compounds of this series are shown in Table 2. From the MIC data it can be derived that these compounds are 20–30 times more active than thiacetazone (*M. lufu:* MIC = 340 μM/l) and 2 to 3 times more active than pyridine-2-aldehyde-TSC. Several compounds recently

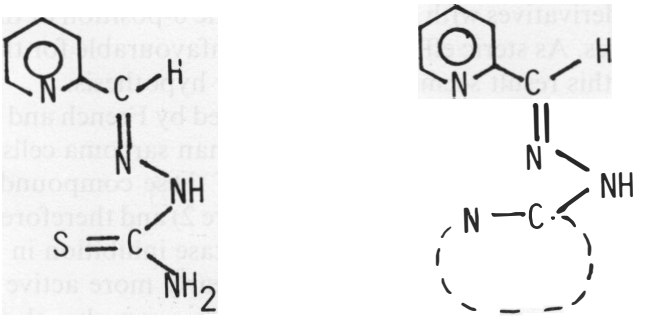

Figure 3. Structural comparison of two tridentate chelators: pyridine-2-aldehyde-TSC (left) and corresponding N-heterocyclic hydrazones of the type of the lead molecule PH22 (right).

Table 2. MIC values (μM/l) of two heterocyclic hydrazones towards mycobacteria

	PH22	PQ22
M. lufu L209	18	10
M. smegm. ATCC607	35	24
M. tbc H37Rv	80	43
M. avium SN304	18	24
M. marinum SN1254	18	12

developed in our laboratory from the new lead molecule PH22 show a further increase in activity by a factor of ten and have MIC values lower than 0·5 μg/ml. Both PH22 and PQ22 are known to be strong tridentate chelators of iron ions which are octahedrally surrounded by two ligand molecules.

As these heterocyclic hydrazones do not provide the possibility to split off hydrogen sulphide they can be expected to be less toxic than pyridine-aldehyde-TSCs. The results of the investigation of the acute toxicity of three compounds towards rats and mice are shown on Table 3. We found that the new lead compound PH22 is considerably less toxic than pyridine-2-aldehyde-TSC and is comparable with thiacetazone in this respect.

A very interesting aspect of hydrazones and TSCs is their synergistic antibacterial effect towards *M. lufu* in a combination with dapsone. By analysing

Table 3. Acute toxicity of PH22 (I), pyridine-2-aldehyde-TSC (II) and thiacetazone (III) towards rats and mice

	LD_{50} [mg/kg]	
(I)	$\geqslant 2000$	rat, subcut.[1]
(II)	30	rat, subcut.[1]
(II)	40	mouse, i.p.[2]
(III)	1000–2000	mouse, subcut.[3]

[1] Kazda, Schaper, unpubl. results

[2] French, Blanz. *Cancer Res*, 1965; **25:** 1454

[3] Bavin, Rees *et al. J Pharm Pharmacol*, 1950; **2:** 764

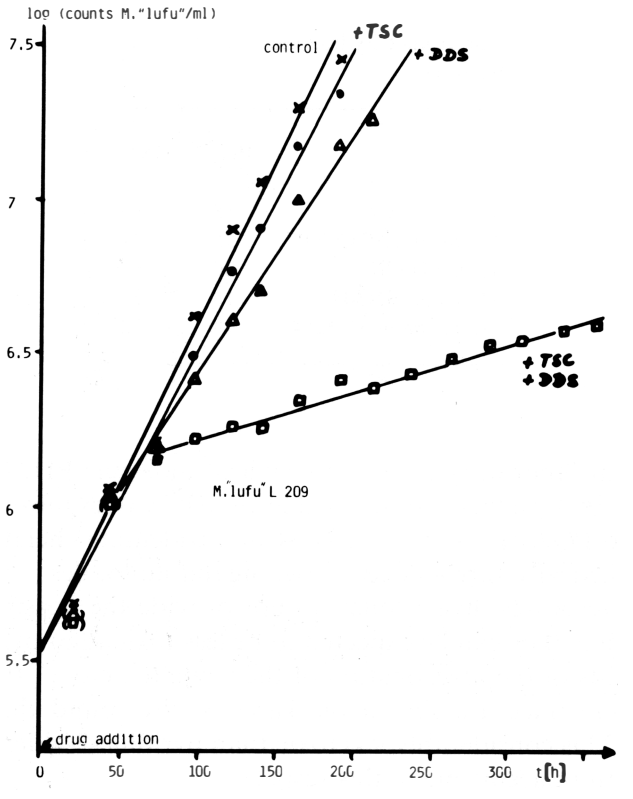

Figure 4. Bacterial growth kinetics of *M. lufu* at 31°C in the presence of pyridine-2-aldehyde-TSC and dapsone (DDS) alone and in combination at the concentrations indicated. x, control; ●, 5 μM/l Py-2-TSC; △, 0·2 μM/l DDS; □, 5 μM/l Py-2-TSC + 0·2 μM/l DDS.

bacterial growth kinetics[13] we found this potentiation of activity for both TSCs and heterocyclic hydrazones. Figure 4 shows that the control growth rate of *M. lufu* is reduced a little bit by low concentrations of pyridine-2-aldehyde-TSC or DDS. If both drugs are combined a clearly more than additive effect is obtained.

A very similar result is obtained if 2 to 3 times lower concentrations of PH22 are combined with DDS (Figure 5).

A possible explanation for the synergism is the fact that the synthesis of DNA is inhibited at two different sites of the DNA pathway: DDS is an inhibitor of folate synthesis[14] whereas the hydrazones seem to block deoxynucleotide synthesis (Figure 6).

One of the subsequent steps in the reaction sequence inhibited by dapsone can be blocked by trimethoprim (TMP) derivatives which are well known DHFR inhibitors.[15] If synergism is obtained by combining hydrazones and DDS then the

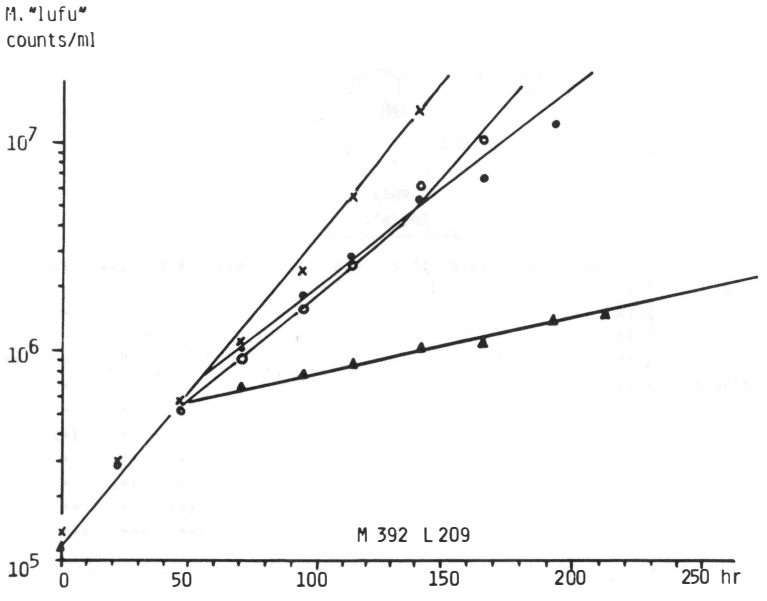

Figure 5. Bacterial growth kinetics of *M. lufu* at 31°C in the presence of PH22 and dapsone (DDS) alone and in combination at the concentrations indicated. x, control; ●, DDS 0·2 μM; ○, PH22 2 μM; ▲, DDS + PH22 0·2 μM + 2 μM.

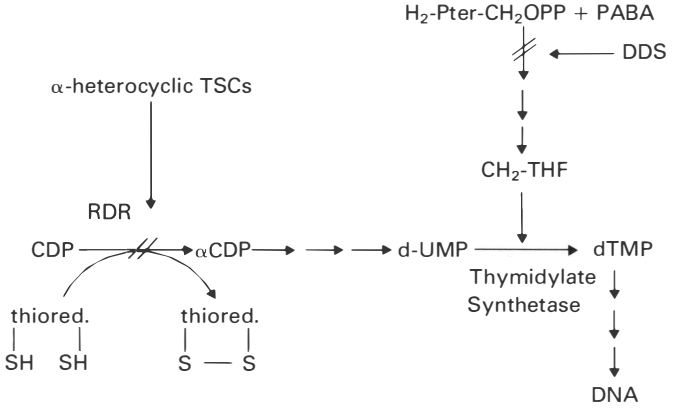

Figure 6. Sites of inhibition of DNA synthesis by sulphones (DDS) and hydrazones e.g. TSCs.

same effect should be observed in a combination of hydrazones and TMP derivatives.

This assumption is confirmed by Figure 7 showing for *M. lufu* the MIC determination by checkerboard titration[16] of combinations of PH22 with the TMP derivative 107-0-2. A clear synergistic effect can be recognized. An increase

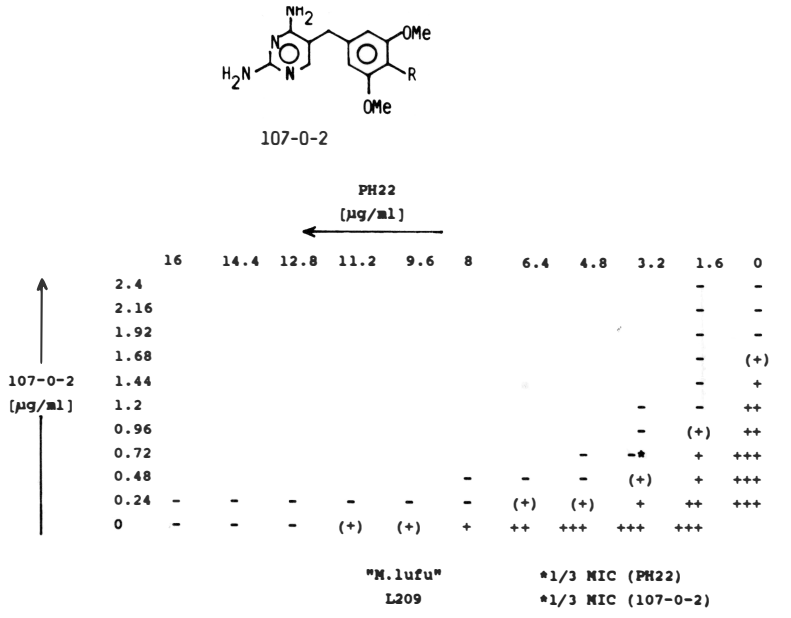

Figure 7. Antibacterial effects of PH22 and TMP derivative 107-0-2 alone and in combination on *M. lufu*. +, multiplication is observed; −, no multiplication observed.

in synergistic potentiation is obtained by combining PH22 with a TMP derivative which is more active towards *M. lufu*.

Fortunately a clear-cut further increase in the synergistic effect is observed (Figure 8) in a triple combination consisting of DDS and of PH22 + TMP 107-0-2 (which are applied in a fixed ratio of 5:1). Here a full stop of bacterial growth is obtained at concentrations of only 2% of the concentrations needed for single drug treatment in case of PH22 and TMP 107-0-2 and 25% in case of DDS.

And last but not least a very strong synergism is found for the combination of PH22 with the DNA synthesis inhibitor 5-fluoro-uracil. This antitumor drug is a blocker of the reaction catalysed by thymidylate synthetase (Figure 6) which promotes the methyl group transfer from methylene-THF to the 5-position of deoxyuridine monophosphate.[17]

So possibly PH22 derivatives are appropriate candidates for a multidrug therapy in leprosy.

The synthesis of DNA in mammalian and microbial cells depends on the rate limiting synthesis of deoxynucleotides which is catalysed by RDR.

As shown by Figure 9 the enzyme consists of two nonidentical subunits B1 and B2.[10] Each subunit by itself is completely inactive. At the active site subunit B2 contains two atoms of iron which are necessary for enzyme activity. Of similar importance is the presence of a tyrosine radical in the same subunit.

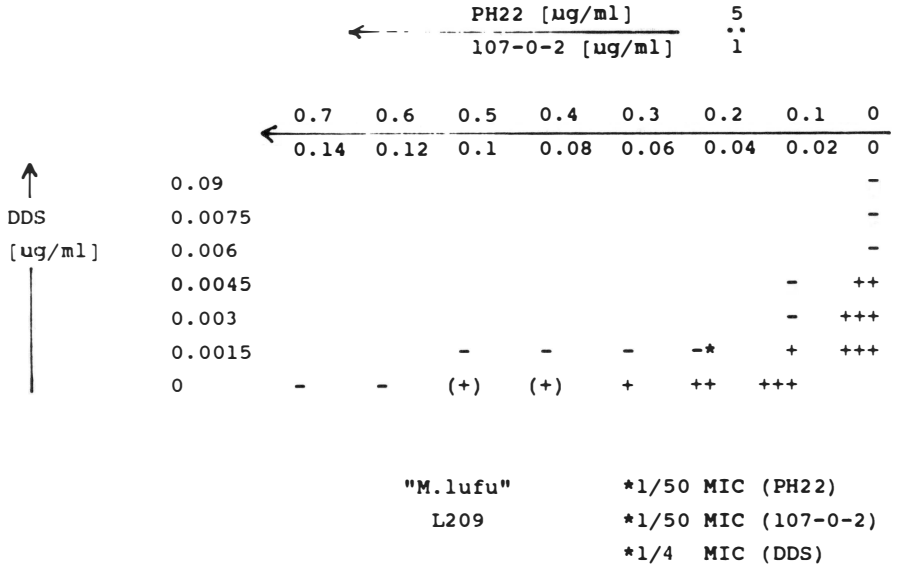

Figure 8. Antibacterial effects of PH22+TMP 107-0-2 (fixed ratio 5:1) and DDS on *M. lufu*.

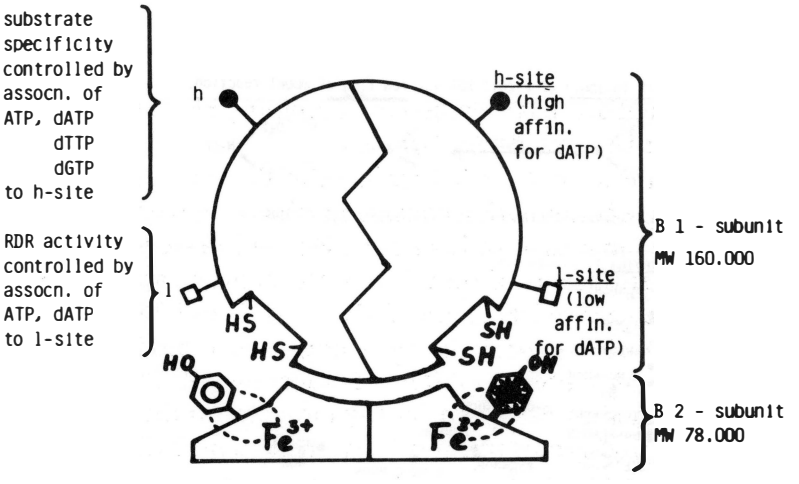

Figure 9. Thelander–Reichard model of ribonucleoside diphosphate reductase (RDR) from *E. coli*.[10]

In a simple chemical model system we tested the chelating properties of some of our derivatives and got for all of them almost identical results. So we had the impression that chelation of the iron atom of ribonucleotide reductase is not the activity determining step.

Several reports can be found in the literature[3,9,18,19] which seem to support this

conclusion and which suggest the active form of the inhibitor to be a preformed iron chelate. Interestingly also some ferrous ion complexes of our chelators were found to be quite active inhibitors of *M. lufu*.

As mentioned before the ribonucleotide reductase in addition to iron atoms also contains one tyrosine radical at the active site. The presence of the radical is closely linked to the presence of iron atoms. The radical is lost on removal of iron and reformed on reconstitution.[20] On the other hand the radical without loss of iron can be destroyed by hydroxylamine derivatives.[11,21]

Thelander found that there is some correlation (Figure 10) between the degree of inhibition of *E. coli* ribonucleotide reductase by N-hydroxyurea analogues and the corresponding rate of reaction of these compounds with an inorganic radical salt (Figure 10), which was considered to be a model system for the tyrosine radical. Thelander concluded that a very important parameter for the inhibitory potency of a drug is the ability to undergo a one-electron oxidation.[11,19]

It is well known that hydrazines and hydrazones are easily oxidizable compounds and that their oxidation often proceeds via radical mechanisms.[22–26] So one can conclude that the hydrazone chelators combine two molecular properties which may be important for activity: 1, the ability to form very stable chelate complexes; this seems to be a necessary prerequisite for activity; and 2, the

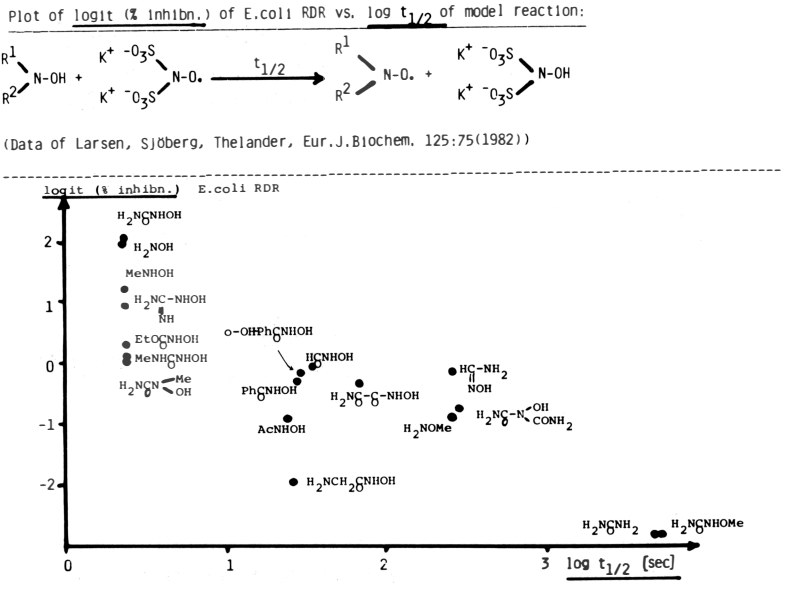

Figure 10. Comparison of inhibitory activity and reactivity of N-OH-urea derivatives; plot of log (% inhibition) of *E. coli* RDR *vs.* half life (log $t\frac{1}{2}$) of the model reaction shown at the top of the figure; data obtained from ref. (11).

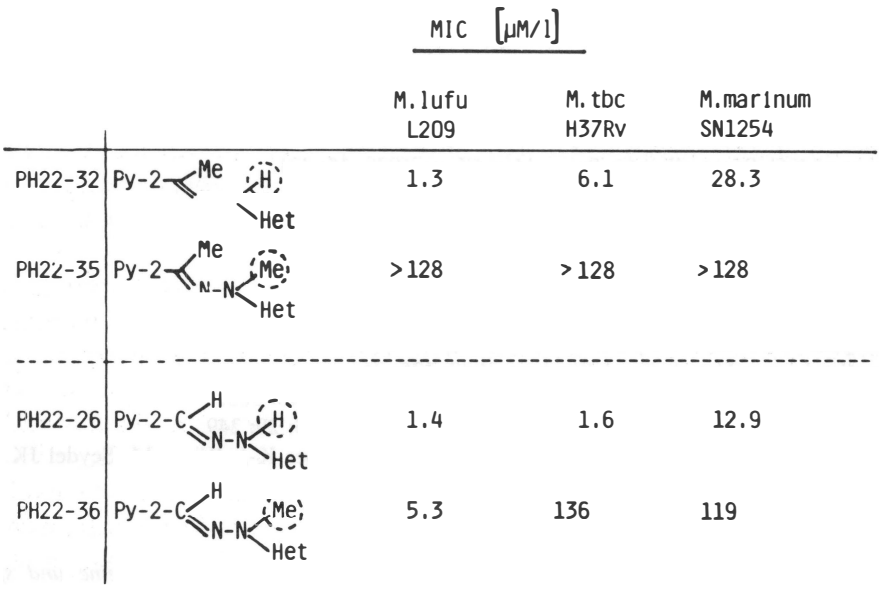

Figure 11. Decrease of antibacterial potency of PH22 derivatives observed after methylation of the hydrazone NH group.

ability to perform redox reactions; possibly the biological potency is modulated by the substituent effects on the postulated radical mechanism.

This last assumption is supported by the results shown by Figure 11. A large decrease in activity is observed if the most probable site of oxidative attack of the heterocyclic hydrazones is blocked by transforming the NH fragment within the hydrazone group into a N–Me group.

On the basis of the lead molecule PH22 we are presently trying to develop a new drug with high activity against leprosy by synthesizing a series of PH22 derivatives and testing their activity against mycobacteria. Unfortunately for patent reasons no details of this work can be given.

By applying quantitative structure–activity relationship analysis[27] we hope to extract relevant information for the attempted activity optimization.

Acknowledgments

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Efficacy of different regimens in multibacillary leprosy

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The necessity for combined chemotherapy in multibacillary leprosy has been recognized for about 15 years.[1–3] It was however entirely unknown how this combined therapy had to be applied, particularly in respect to the drugs to be given, the frequency of their administration and above all the duration of therapy.

We have approached the problem on the basis of results obtained in experimental chemotherapy of *Mycobacterium leprae* infections in mice,[4–12] observations made in man[13–15] and prospective chemotherapeutic trials in patients.[16–19]

From studies in mice and man, we know that there are at present two bactericidal drugs: rifampicin (RMP) and the thioamides (either ethio- or prothionamide, ETH or PRO), and two mainly bacteriostatic drugs: dapsone (DDS) and clofazimine (CLO). RMP is extremely bactericidal, killing about 5 logs of *M. leprae* after a single dose of 1500 mg or a few doses of 600 mg. Due to its profound effect on *M. leprae*, it can also be given intermittently up to once a month[8] whereas ETH or PRO when administered less than three times a week, are inactive.[9,10]

Dapsone and clofazimine are interchangeable as bacteriostatic drugs, and since they have a different action mechanism they do not show cross-resistance.

From what was known in the treatment of tuberculosis, we concluded that what was needed for the treatment of MB leprosy was[1,2] an association of two and preferably three, bactericidal drugs (but only two are available in leprosy) in order to obtain maximal killing of *M. leprae*; and the addition of a bacteriostatic drug, whose main role would be to prevent the selection of organisms resistant to the bactericidal drugs.

In this interpretation of the events occurring during chemotherapy, the use of a second bacteriostatic drug was unnecessary. We therefore used in all our trials DDS in new patients and replaced it by CLO in patients at risk for secondary DDS-resistance, defined as those patients who had been treated with DDS for 5

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years or more. (The importance of primary DDS resistance was unknown when we started our studies.)

We accepted as an unproven premise, that daily administration of RMP had a greater bactericidal effect than intermittent administration. Although we had found that, with the Morphological Index as a measure, there was no difference between daily 600 and weekly 900 mg of RMP[16] and with relapse as a measure[15] two doses of 600 mg RMP per week were sufficient. Thus it was clear that in the association ETH-RMP, the latter could be given intermittently but with what intervals remained to be investigated.

As a conservative measure the first regimens studied contained daily administration of the combination RMP–ETH. In later trials RMP was given intermittently after an introductory phase of daily RMP.

The question of the duration of therapy has many facets. A particular aspect to be fully realized in the treatment of leprosy, is the dissociation between bacterial killing and skin smear status: *M. leprae* can be rapidly killed by appropriate bactericidal drugs but the dead bacilli are extremely slowly eliminated by the host. This means that the Bacterial Index cannot be used as a measure of cure—only the eventual appearance of relapse is a reliable criterion.

Thus the question concerning the moment of appearance of relapses becomes crucial, since this will define the duration of the follow-up period after the end of treatment and will influence greatly the time scale for prospective treatment trials. Our observations showed that 50% of relapses in MB disease appear within 2 years and 50% of relapses in PB leprosy within 3 years after the end of therapy.[20,21] We thus proceeded on the premises that antibacterial treatment can be stopped in the presence of a positive BI, and that the measure for cure is the absence of relapse, being documented by congruent clinical, bacteriological (MI!) histopathological information, together with the result of mouse footpad inoculation of bacteria from a skin biopsy.

The duration of treatment can only be defined by trial and error. We decided to proceed stepwise downward from 1 year. This 1-year period was not entirely arbitrary: in the treatment of tuberculosis, regimens comprising two bactericidals were highly effective when applied during 9–12 months.

The following 12 month regimens were tested in Burundi and on the island of Anjouan, Republic of the Comores[17]:

8—44 RED
8W RMP 600 7/7 + 44 W RMP 600 1/7
52W ETH 500 7/7
52W DDS 100 7/7

8–44 REC
8W RMP 600 7/7 + 44 W RMP 600 1/7
52W ETH 500 7/7
52W CLO 100 7/7

The REC regimen was given to patients at risk of or with proven secondary DDS resistance.[23] The results are presented in Table 1. It may be concluded from these results that MB leprosy can be cured by a treatment regimen of 1-year duration. However these regimens are difficult to apply on a large scale. It was therefore decided to start another trial in which the supervised treatment was reduced to 6 months. In Zaire and Rwanda the following regimens were applied:

RED/ED or RED/D

26W RMP 600 7/7, ETH 500 7/7, DDS 100 7/7 } +26 W ETH 500 7/7, DDS 100 7/7 or (randomization) +26 W ETH 500 7/7, CLO 100 7/7

or REC/EC or REC/C

26W RMP 600 7/7, ETH 500 7/7, CLO 100 7/7 } +26 W ETH 500 7/7; CLO 100 7/7 or (randomization) +26W CLO 100 7/7

Regimen RED was administered to all newly diagnosed male patients and all male patients treated previously for less than 5 years. They were randomized between RED/ED and RED/D. Regimen REC was administered to all female patients and all male patients treated previously for 5 years or more. They were randomized between regimens REC/EC and REC/C.

The results are presented in Table 2, and the confidence limits for the different groups and regimens in Table 3.

The results of this study show that it is possible to cure MB leprosy by a

Table 1. Follow-up of multibacillary patients in Anjouan and Burundi

	Previously untreated	Previously treated	Previously treated and DDS–R
	8—44 RED	8–44 RED	8–44 REC
Total	98	59	21
Died	3	2	1
Lost	4	—	3
	91	57	17
2 yr	89	54	17
3 yr	23/25(×)	25/28	4/4

Number of patients with 3 year follow-up/number of patients taken in. Upper 95% confidence limits for absence of relapses after 2 and 3 years. RED in new patients: 0/89 = 4·0% RED in new and old patients: 0/143 = 2·50%. Overall 0/160 = 2·28%

Table 2. Number of patients in each category and treatment groups and their follow-up.

	NC				OC			
	RED/D	RED/ED	REC/C	REC/EC	RED/D	RED/ED	REC/C	REC/EC
Total	27	37	22	11	10	6	67	62
Lost	3	8	4	2	2	—	7	4
Excl.	—	1	1	—	—	—	3	2
Died	—	4	—	—	1	1	5	12
	24	24	17	9	7	5	51	44
2 yr	23	15	14	6	6(+1)	4	44	36
3 yr*	6/11	3/6	4/6	2/2	3/3	2/2	19/25	15/21

NC, new cases; OC, old cases.

Excluded, treatment stopped because of toxic hepatitis.

Yr, years after the end of therapy.

* Numerator: number of patients seen at 3 years after the end of treatment, denominator: number of patients taken in the study during 1981.

(+1), 1 pt not seen at 2 years, was so at 3 years

Table 3. Upper 95% confidence limits for the different treatment regimens of MB leprosy at 2 years after the end of treatment.

a.	RED/D	NC	0/23	14·8%
b.	REC/C	NC	0/14	
c.	RED/ED	NC	0/15	
d.	REC/EC	NC	0/9	
e.	RED/D	OC	0/7	
f.	REC/C	OC	0/44	8·0%
g.	RED/ED	OC	0/4	
h.	REC/EC	OC	0/36	9·7%
	Total		0/152 =	2·4%
a+b:			0/37 =	9·4%
c+d:			0/24 =	14·2%
e+f:			0/51 =	6·2%
g+h:			0/40 =	8·8%
a+b+c+d			0/61 =	5·8%

treatment regimen of 1-year duration, during which rifampicin is given during the first 6 months only. Since RMP is the main bactericidal, and since there is no difference between the administration of DDS or CLO with and without ETH during the second semester, it seems that ETH has little effect. The necessity of any additional treatment after 6 months of a powerful bactericidal combination may even be questioned.

Therefore a regimen of 6 months duration was tested. Such a regimen was applied in Istanbul in collaboration with T. Saylan *et al.*[19]

2W	RMP 600 7/7 + 24W	RMP 600 1/7
	ETH 500 7/7	ETH 500 7/7
	DDS 100 7/7	DDS 100 7/7

(With DDS replaced by CLO in patients treated for 5 years or more with DDS).

Unfortunately only a small number of patients could be followed for 2 years after the end of treatment, widening the confidence limit to 11·9%.

The above regimens were also accompanied by a serious and unforeseen complication, namely toxic hepatitis due to the association RMP-ETH. It occurs in 3·5–5% of patients, after 5–186 days of treatment, with a mean of 93 days and a median of 76 days, whether the combination RMP–ETH is given daily throughout or only during an introductory phase of 2–8 weeks.

In the meantime results of a still shorter drug regimen of 3 months duration have become available. The regimen is:

13W	RMP 600 2/7
	ETH 500 7/7
	DDS 100 7/7 (or CLO 100 7/7)

Sixty-four patients were taken into the trial. From each of them $5{\cdot}10^3$ bacilli from the skin were inoculated into mouse footpads in Antwerp to ascertain the viability of the organisms.

Bacilli from 33 patients proved infectious for mice. These patients were all bacteriologically positive at the end of therapy. Biopsies from patients have again been inoculated into mouse footpads at 2 years follow-up. No viable bacilli could be detected. The patients have been followed for the third year, none of them has shown any sign of relapse. Furthermore, not a single case of hepatitis has been observed. This may be the result of several concurrent factors:

1 Considerable shortening of the duration of treatment: whereas toxic hepatitis manifests after a median treatment duration of 93 days, a 90-day regimen should eliminate 50% of hepatitis cases.
2 RMP is administered intermittently twice a week from the very start of treatment.
3 The small number of patients in this study (39). However a total of 61 were

Table 4. Comparison of four treatment regimens in multibacillary leprosy

Regimens		Duration in weeks: treatment	Duration in weeks: RMP administration	Doses of RMP	Number of new patients	CI†
8–44 RED(C)	8w 6/7 44w 1/7	52	52	92	89	4%
RED/D(E) C(E)	26w 6/7 26w 6/7	52	26	156	48	7·4%
2E7D7 24R1E7D7		26	26	36	23	12%
13R2E7D7		13	13	26	33*	10·6%

* Patients whose bacilli in the skin were infections for mouse footpads
† CI, upper 95% confidence interval.

included, 22 of them with negative mouse footpad results, and not included in the final analysis of the results of the antibacterial treatment.

In conclusion MB leprosy can be cured by regimens of 12, 6 and even 3 months duration.

Table 4 shows the confidence limits of the results obtained in relation to the number of doses of RMP administered and the duration of therapy. Daily administration of RMP is not a necessity thus invalidating one of our starting premises. Depending on the regimens, twice or even once weekly administrations are sufficient, thus improving also on the toxicity.

It seems that as in experimental chemotherapy in the mouse there is a relationship between the dosage of RMP, the intermittency and the duration of treatment, allowing for widely different regimens in terms of the total dose of RMP administered and the duration of treatment. Thus regimens could be devised that are adapted to widely different local situations.

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Preliminary results of treatment of leprosy patients in The Netherlands with daily rifampicin, dapsone and clofazimine

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Since 1979 all leprosy patients in the main treatment centres in The Netherlands have been treated with a multiple drug regimen. Pre-treated nonlepromatous patients received 600 mg rifampicin and 100 mg dapsone daily for 6 months. In previously untreated patients dapsone treatment was continued for $1\frac{1}{2}$ years more. For several reasons the combination of rifampicin and Isoprodian was not used: Isoprodian is not registered in The Netherlands and to obtain registration would have been time consuming and difficult. Previous experiences with prothionamide in drug combinations were not encouraging. Out of 15 patients, 3 developed hepatitis, in 2 cases a severe hepatitis.

It was thought to be interesting to test another drug combination in a situation where the chance of reinfection is remote. A combination without prothionamide and isoniazid would be interesting for comparison with the Malta regimen and a regimen including daily rifampicin would be interesting for comparison with the WHO Study Group regimen.

About 400 patients, nearly one-third being lepromatous or borderline lepromatous, have completed the course of multiple drug therapy and have been followed up clinically and serologically for 3–6 years. Most lepromatous patients have been without treatment for 3–5 years, most nonlepromatous patients for 4–6 years. Until one month ago no relapses were seen. Then a lepromatous patient who had completed MDT, reported with a single, small nodule on his wrist. The serological test with monoclonal antibodies against *Mycobacterium leprae* specific phenolic glycolipids showed levels which were as high as seen in active lepromatous leprosy.

Relapse was confirmed by a biopsy, showing BI 5+, with 27% nongranular bacilli. The patient had been treated with dapsone since 1957 and a first relapse occurred in 1972. Combined treatment was started in February 1980 and treatment was withdrawn in February 1981. In 1980 no bacilli were found in a biopsy specimen. The second relapse occurred 5 years after cessation of treatment.

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The present report is preliminary. A more detailed analysis will be made when all patients have completed a period of at least 5–6 years without treatment. In many aspects the patient material in The Netherlands is largely comparable with the patients in Malta. A very high proportion of the patients had received monotherapy for many years, prior to MDT. Several patients were still bacteriologically positive when MDT was started and after cesation of chemotherapy.

It is of interest that so far only one more patient has shown similar high values in the serological tests with monoclonal antibodies against phenolic glycolipids. As yet no clinical evidence of relapse was found in this patient.

The only conclusion which can be drawn at present is that after a follow up period of about 4 years after cessation of treatment, the relapse rate is not significantly higher than after treatment with rifampicin and Isoprodian. The absence of high values in the serological tests suggests that the relapse rate may well remain very low for a longer period, but this will have to be confirmed by a longer follow up period.

At present there are as yet no data available for a comparison of relapse rates with the WHO Study Group regimen.

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Final bulletin

D L LEIKER, A M DHOPLE & ENNO FREERKSEN

In the sixties it became obvious that the era of successful monotherapy with dapsone would soon come to an end, due to worldwide increasing emergence of sulphone resistance of *Mycobacterium leprae*. Since then the innovating concepts of Professor Freerksen have become the basis for a new approach in the treatment of leprosy. Based on his experience in tuberculosis he called for multidrug therapy in leprosy as in tuberculosis with therapy of limited duration, for shorter periods than in the sulphone era. He took into account the element of human weakness in taking drugs and introduced the first combined formula with all drugs in a single tablet, undoubtedly a realistic measure to prevent the intake of only one drug by a patient, which would lead to resistance to this drug.

For the first time detailed information, including independent assessments of the Malta Leprosy Eradication Project, were made public. The results, after a follow-up period of 10 years after release from treatment, are by all standards excellent.

Although the drug combination proposed by Professor Freerksen are not accepted by everyone, his principles for chemotherapy are now generally accepted. The Malta experiences formed the basis for the recommendations in 1982 by WHO Study Group on Chemotherapy of chemotherapy.

It is now generally accepted that MDT is the only answer to the emergence of drug resistance. We may expect a higher rate of cure after a shorter treatment period than before. If MDT is properly applied we may expect that it will bring the control of leprosy a major step nearer.

We have listened to reports on the use of rifampicin–Isoprodian in a leprosy and TB control project in Paraguay; the results were favourable. We have listened to reports on experiences with the WHO Study Group regimen from India, Malawi, Nepal, Ethiopia, Tanzania, Sierra Leone and the Dominican Republic. These reports show that in spite of many difficulties which must be overcome, MDT is operationally feasible also in situations where the infrastructure is less favourable than in Malta. The great challenge is now to apply MDT worldwide. Close cooperation between governments and voluntary agencies specializing in

leprosy aid, such as the German Leprosy Relief Association, will be a necessity to reach this goal.

A randomized multicentre study did not reveal measurable differences between a dapsone monotherapy regimen, a combination of dapsone and rifampicin and rifampicin plus Isoprodian, but this applies only to the treatment phase. Time must teach what will happen hereafter.

In Turkey a short course MDT regimen of only 6 months resulted in no relapses in 29 MB patients after a follow-up period of 2–3 years.

In the Netherlands about 400 patients, one third MB patients, were treated for 1 year with daily rifampicin, dapsone and clofazimine on alternate days. During a follow-up period of 3–5 years only one relapse was seen, the results are not significantly different from those in Malta.

In Malta morphologically intact bacilli were found in some patients, without clinical evidence of relapse. In Paraguay few relapses were recorded. This suggests that none of the present regimens is capable of eliminating all persisters. However, precise information about the significance of small numbers of persisters for the risk of relapse is still lacking.

The conference shows that although we are now beginning to see clearly the direction in which we have to go for controlling leprosy, we still have much to learn about the exact way.

Little is still known about the minimum duration of treatment required for obtaining acceptably low relapse rates with the different regimens.

The reasons for finding little toxicity after regimens with PTH in several areas and significant toxicity in others is yet not clarified.

Seemingly simple questions such as reliable criteria for distinguishing between relapse and reversal reaction which are essential for assessing relapse rates, are not yet available.

During discussions a strong point was made of the need for standardizations of definitions. We must learn from all MDT projects. Each project is in fact a trial. But we can learn only when authors state in their reports their meaning of definitions used.

Earlier I had said that we can expect much from MDT if it is properly applied with the emphasis on properly. But will this really happen? I doubt it very much. Drugs are available on the black market or without a prescription in pharmacies. Drug abuse cannot be entirely avoided.

Therefore in the long run there remains the threat of emergence of drug resistance, in particular to rifampicin. This brings me to the second part of the conference.

In addition to making use of the presently available tools in the best possible way there is an urgent need for development of new tools. Strengthening a fundamental research deserves a high priority. Since research is time-consuming and it takes many years before the fruits of research have been tested and can be applied in the control of the disease we cannot afford delays.

THE SECOND HALF OF THE CONFERENCE DEALT WITH THESE SUBJECTS

The inability to cultivate *M. leprae*, the causative agent of human leprosy, has been a bottle-neck in leprosy research since its discovery in 1872 by Armauer Hansen. However, some significant progress was reported during this Symposium. Along with microscopic counting of acid-fast bacteria in cultures, various biochemical parameters were used to evaluate the *in vitro* growth of *M. leprae*. The primary isolates retained all the characteristic of *M. leprae* even though subcultures could not be achieved. The old theory of *M. leprae* being a 'microbe-dependent microbe' was again brought to the surface.

Environmentally derived mycobacteria were isolated from soil samples collected from leprosy endemic areas and were identified as resembling *M. leprae*. This was confirmed by inoculating into footpads of normal and nude mice. However, it was suggested to differentiate soil-derived cultivable mycobacteria from *M. leprae*.

There is an urgent need to develop newer antileprosy compounds for use in endemic areas. It was suggested to employ metabolic activities in *M. leprae*, which are essential for growth and survival of bacteria, as possible targets of antileprosy agents. Such targets can be cell wall synthesis and proteins synthesis. Nucleic acid synthesis seems to be another target and two known antileprosy agents, rifampicin and clofazimine, appear to affect these pathways.

The most significant contribution of this Symposium was the presentations on *in vitro* test systems for rapid screening of potential antileprosy compounds. One method was the *in vitro* culture system where biochemical parameters were used to evaluate the effects of drugs on *M. leprae*. Another was the use of hypoxanthine incorporation by *M. leprae*. In other test systems *M. leprae* were phygocytosed by human and mice macrophages and the effects of drugs on *M. leprae* were evaluated inside these macrophages using various test systems. These systems include Fe receptor assay using EA rosetting technique, fluorescence staining using fluorecin diacetate, uracil uptake assay and sialic acid assay. In all these test systems effects of known antileprosy compounds were tested to offer assurance about the feasibility of these systems and encouraging results on the efficacy of some newer compounds on the viability of *M. leprae* were presented. High technology instrumentation has also been employed to assess the viability of *M. leprae*. This dealt with the use of mass spectrometric analysis of single *M. leprae* cells obtained from the biopsies of patients under chemotherapy. The information is derived from the intracellular concentration of sodium and potassium ions and also from fragment ions of the complex cell matrix. Excellent correlation was described between the results obtained with this technique and those obtained by ATP assay and mouse footpad assay.

The current feeling is that leprologists will accept these systems provided it can be shown that these results are parallel to those obtained by the mouse footpad model. So the mouse footpad system was described in full detail and

explanations on continuous method, kinetic method and proportional bactericidal method were offered. It was further reported that the kinetic method might be most suitable since this method distinguishes bactericidal drugs from bacteriostatic drugs. With this method several new classes of compounds have been tested. It was also reported that drug monitoring trials can be undertaken utilizing immunologically suppressed mice and rats including nude rats.

It seems the laboratory investigators are moving in a proper direction and that such studies should receive constant encouragement from all concerned with leprosy.

The new routes for the development and screening of potential antileprotic drugs and drug combinations. The new compounds were synthesized with certain enzymes of bacteria as targets. The best example being the dihydrofolate reductase of both *M. lufu* and *M. leprae*. Both whole cells and cell-free enzymes were used. Several trimethoprim derivatives were synthesized and two compounds namly K-130 and Brodimoprim were found to be effective against both the sytems of both bacteria. Furthermore, synergistic activities were also observed with these compounds. Another target enzyme used was ribonucleotide reductase to evaluate the effects of thiosemicarbozones.

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ADDENDUM

Multidrug therapy cost: a hypothetical analysis

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Introduction

Multidrug therapy (MDT) has been widely accepted as an effective intervention tool for the time being in leprosy control programmes to overcome problems like dapsone resistance, drug persistors and poor drug compliance for long-term treatment. Various authentic bodies like WHO,[23] ILEP,[11] Indian Association of Leprologists (IAL)[12] and Borstel[2] recommended their drug regimens to be suitable for all situations of leprosy control programmes. Recently another drug regimen called Isoprodian–RMP (ISO–R—rifampicin is combined in Isoprodian) has been introduced on a multicentre trial basis by the German Leprosy Relief Association.[8] Pattyn *et al.*[17] recommended a regimen for developed countries similar to WHO, but advocated administration of daily rifampicin instead of a monthly pulse. A lot of experience has been gained over the past 3 years in using different regimens in field conditions, and preliminary reports are available on the efficacy of drug combinations and operational aspects of WHO–ILEP and IAL regimens.[9,10,15,18,19,20] Effectiveness of Isoprodian with rifampicin (Borstal therapy) in a mass scale Malta-Project has been reported[4] and subsequent long-term follow up evaluation has been reported recently.[7,14] No such data are yet available on Isoprodian–RMP (ISO–R) and other regimen. Whatever type of regimen one is likely to implement for a mass scale programme to meet the objectives, in addition to efficacy, feasibility, acceptability, availability of drugs, it is very essential to consider the cost factor of both drug and operational costs of mass scale multidrug therapy programmes in view of financial constraints. This is essential as we do not have definite data on the most effective drug regimen for a short duration with least toxicity meeting the objectives of multidrugs, period required for curing cases, relapse rates and other epidemiological data to launch a global leprosy eradication programme, which will be essential to eradicate leprosy from any country as man becomes a reservoir of infection whose movement cannot be stopped.

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No reports are yet available on the cost-effectiveness of MDT programmes except one report,[1] where this has been worked out in comparison to DDS monotherapy for 5 years which is to be considered as tentative. As the necessary data are not available on MDT to work out cost-effectiveness, only the drug cost is worked out.

Hypothetical basis for cost factor workout

To work out the cost factors in all the above mentioned regimens for a mass programme, it is presumed that all the regimens would be effective in both multi- and paucibacillary leprosy, as all these combinations have rifampicin which is a strong bactericidal drug. It is also presumed that all these regimens are well accepted and tolerated (all these regimens are being used by this project, where it is observed that the majority of patients under different groups tolerate these well). With these presumptions, drug cost has been worked out for currently available multidrug combinations for mass scale implementation in a hyperendemic situation where prevalance rate is 10 and above per thousand population.

For calculation purpose it is presumed that multidrug therapy has been started in a population of 4,000,000 and equally divided for the following four regimens with a prevalance rate of 10 per thousand. 1000 cases are expected for each group. 1, Isroprodian–RMP (ISO–R); 2, Borstel; 3, WHO/ILEP; 4, IAL.

Cost of the drugs (in Indian currency—approximate)

As Isoprodian and Isoprodian–RMP is not yet available in the Indian market, the cost per tablet has been worked out exclusively on a proforma invoice sent by Saarstickstoff–Fatol GMBH 6685 Schiffweller, Federal Republic of Germany and the value shown in DM has been converted into Indian rupees. As the rest of the drugs are available in India, the cost is worked out on current rates: Isoprodian, Rs. 0.22 per tablet; Isoprodian–RMP (ISO–R), Rs. 0.25 per tablet; rifampicin (150 mg), Rs. 0.80 per capsule; clofazimine, Rs. 0.70 per capsule; DDS (100 mg), Rs. 0.05 per tablet.

Estimation of caseload

For chemotherapy purposes WHO classifies all the LL, BL, BB patients with $\geqslant 2$ BI as multibacillary and the rest as paucibacillary. (I, TT, BT).[22] However, in view of difficulties in the programmes in developing countries fore reliable smear reports with Ridley's scale, the Indian Association of Leprologists group simplified this classification and designated all the positive smears (LL, BL, BB)

Table 1. Regimen cost analysis per patient (Indian currency)

	ISO–R (GLRA)	Isoprodian –rifampicin (Borstel)	WHO–ILEP	IAL
Multibacillary (2 years)				
Adult	720.00	2628.00	718.00	825.00
Child	360.00	1314.00	359.00	413.00
Paucibacillary (6 months)				
Adult	180.00	657.00	28.00	28.00
Child	90.00	329.00	14.00	14.00

Cost is worked out for a minimum period of 2 years in multibacillary cases and 6 months in paucibacillary cases as per WHO and recommendation.[22]

as multibacillary and the negative smears (I, TT, BT) as paucibacillary.[12] Hence this criteria hs been applied for this estimation.

The following data obtained from the Bombay Leprosy Project field were collected over the past few years to work out the caseload (though it was not statistically collected data):

Based on the above figures (Table 2), the following figures have been worked out.

Prevalence rate: 10 and above per thousand population.

Population covered under multidrug therapy: 1,000,000 in each regimen.

Table 2. Age and type distribution of leprosy cases.

Age group	Total	Multibacillary (MB)*	Paucibacillary (PB)†	Paucibacillary* (multilesional)‡
Adult	2948 (70%)	371 (13%)	2577 (87%)	2061 (80%)
Child	1321 (30%)	19 (2%)	1302 (88%)	260 (20%)
Total	4269	390	3879	2321

* Multibacillary type (smear positive): adult, 13% of total adult cases; child, 2% of total child cases.

† Paucibacillary type (smear negative): adult, 87% of total adult cases; child, 98% of total child cases.

‡ Paucibacillary type with 4 and more lesions: adult, 80% of adult paucibacillary cases; child, 20% of child paucibacillary cases.

Table 3. Age and type caseload.

Age group	Total no. of cases	Multibacillary (MB)	Paucibacillary (PB)	Paucibacillary with 4 and more lesions
Adult	700	91	609	240
Child	300	6	294	60
Total	1000	97	903	300

Total number of patients: 1000 in each group.

Table 4 shows the total cost of the drugs used in different regimen to complete minimum period of 2 years and 6 months of adequate treatment of multi and paucibacillary leprosy cases respectively, as per WHO recommendation.[22] No operational cost is included. The new cases occurring during the subsequent years, and deletion due to various reasons are not considered for this calculations as sufficient data is not available under each drug regimen. Cost of treating multilesional paucibacillary cases like multibacillary patients is not considered to workout cost, which will definitely increase the size of the budget.

1 The drug cost analysis showed that out of all the regimen recommended for control programmes WHO–ILEP regimen worked out to be cheaper for completing minimum treatment of 2 years and 6 months in multibacillary and paucibacillary types respectively.

2 Initial 21 days intensive therapy with rifampicin in IAL regimen increases the cost. The advantage of this initial therapy is not clearly established. However, a recent preliminary investigation where untreated high multibacillary types treated with WHO and IAL schedules did not show any significant difference in

Table 4. Drug cost in different regimen (Indian Rupees)

	Isoprodian–RMP (GLRA)	Isoprodian + rifampicin (Borstel)	WHO/ILEP	IAL
Multibacillary (2 years)				
Adult (91 cases)	65,520.00	239,148.00	65,338.00	75,075.00
Child (6 cases)	2,160.00	7,884.00	2,154.00	2,478.00
Paucibacillary (6 months)				
Adult (609 cases)	109,620.00	100,113.00	17,052.00	17,052.00
Child (294 cases)	26,460.00	96,726.00	4,116.00	4,116.00
Total	203,760.00	743,871.00	88,660.00	98,721.00

loss of viability of *Mycobacterium leprae* demonstrated by mouse footpad and *in vitro* techniques.[21] However this is to be confirmed on a larger sample. An analysis of bacteriological conversion rate in 146 (IAL schedule) and 103 (WHO schedule) patients in the Bombay Leprosy Project after 24 pulse doses showed that 59 (40%) and 64 (62%) bacterial negativity in IAL and WHO group respectively. These findings question the need for intensive therapy in IAL schedule as well as usefulness of daily rifampicin in Borstel and Isoprodian–RMP (ISO–R) combination, which increases the cost.

3 However the Malta Project where Borstel therapy was implemented and long term follow up for more than 10 years showed no relapses and occurrence of new cases except in 10 cases where solid bacilli could be demonstrated in finger smears which are considered as persisters.[14] Such longitudinal population based studies would be essential in other regimens also to study relapse rate, incidence rate and need to continue treatment till smear negativity before any schedule is accepted as ideal even from the point of cost which is one of the important constraints in any control programme, especially for voluntary agencies.

4 Isoprodian along with rifampicin is also found to be effective in tuberculosis.[5] This would be an added advantage for mass chemotherapy programmes using Isoprodian containing regimen where pulmonary tuberculosis cannot be ruled out on a mass scale; which has to be done if monthly pulse therapy of rifampicin is to be administered or associated tuberculosis is to be treated. The combined chemotherapy programme for tuberculosis and leprosy in Paraguay[6] and Tanzania[16] may evaluate effectiveness of Isoprodian combinations in the light of cost factor in areas where both these diseases are endemic.

5 Supervised administration of rifampicin has been advocated to prevent irregular intake of this drug and emergency of rifampicin resistant strains. In the Malta Project, where daily rifampicin was administered without supervison, no case emerging with resistance to any one of the drugs included in Borstel therapy has been reported so far. On the contrary intermittent administration of rifampicin may give rise to adaptive resistance.[3] Long term surveillance of cases included under WHO–IAL therapy may provide useful data on this aspect.

6 To monitor drug intake (compliance) a simple test like 'Tile test' has been devised for field conditions.[13] This test monitors only the intake of DDS. However for rifampicin and clofazimine, no such simple tests have been devised. In this respect the drug ISO–R will have an added advantage, i.e. if tile tests can detect DDS in urine, it indicates that the patient has swallowed all four drugs. Similarly in the case of Isoprodian the patient swallows three drugs including prothionamide which is also a strong bactericidal drug.

Ultimately it is left to the programme managers to choose any type of drug regimen recommended by expert groups for eradication of leprosy and/or tuberculosis (if a combined programme is operated) considering cost effectiveness of any drug combination over number of years of programme. This has been

rightly pointed out by Askew[1] and Freerksen and Rosenfeld[5] comparing multidrug therapy programme to monotherapy with DDS.

Longitudinal population based studies with different drug regimen are required for comparative evaluation of these drug regimen with respect to cost effectiveness. However in the meantime the available data on these different schedules could be compared from the point of bacteriological conversion rate in multibacillary types and clinical response in paucibacillary type. In addition to this comparative data on toxicity and incidence of reaction is also essential.

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Discussions

Copies of discussions are available on request by writing to: DAHW, D-8700 Würzburg 11, Dominikanerplatz 4, Germany. Please state which papers you are interested in.

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