Resistance to dapsone and rifampin in *Mycobacterium leprae* isolated from leprosy patients of São Paulo State

Resistência a dapsona e rifampicina em *Mycobacterium leprae* isolado de pacientes portadores de hanseníase no Estado de São Paulo

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Abstract

In 1981, reports of resistance to dapsone and rifampin led WHO to recommend multidrugtherapy in leprosy treatment. One of its main goals is preventing the selection of drug-resistant strains of mutant bacteria. Dapsone was the first drug which was experimentally proved to induce resistance; this became possible only after the mouse footpad inoculation technique was standardized, in 1960. There are some important criteria to suspect the existence of resistance, such as: relapses in multibacillary patients already treated or undergoing treatment or unsatisfactory clinical response. Our study aimed at detection of dapsone and rifampin resistant strains among 40 treated patients with clinical signs of relapse, from different cities of São Paulo State and its capital, employing the mouse footpad inoculation. We detected dapsone resistant *bacilli* in 11 cases. Among them, 5 showed total resistance, 1 intermediary resistance and 5 partial resistance. Rifampin resistant *bacilli* were detected in only two cases. We did not detect any case of multiple resistance. The high level of resistance to dapsone is probably a consequence of many years of sulfone and sulfone derivative monotherapy. As for the rifampin, the drug was probably irregularly used in monotherapy; or the patient may have used it previously to treat another disease. The detection of resistant *bacilli* in patients who do not show any clinical improvement, or relapse after treatment, is an important matter to be considered in the future prevention of new cases of resistance. The emergence of resistant strains, especially to rifampin, as well as its dissemination, may cause difficulties to the patients's treatment and jeopardize the leprosy control programs.

Key words: *Mycobacterium leprae*; resistance; dapsone; rifampin

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Introduction

Several reports of secondary and primary resistance to dapsone (DDS) and also to rifampin (RMP) led the World Health Organization (WHO) to recommend, in 1981, a new therapeutic scheme: the multidrugtherapy (MDT). This scheme should not only cure the patient and avoid the development of incapacities, but also prevent the selection of drug resistant mutant strains, especially among multibacillary patients (MB)[1]. With a partially supervised scheme and fixed doses, MDT has contributed in a remarkable way do the disease's cure, reducing prevalence rates in almost 90%. In the beginning of 2004, there were 460,000 patients under treatment all over the world[2]. Despite the demonstrated efficacy of this scheme, it could not avoid the appearance of strains resistant to some of its drugs, even many years after of its introduction.

In respect to epidemiology, there are two cases of resistance: secondary or acquired resistance, resulting from inadequate treatment and generally accompanied of initial clinical improvement, and subsequent worsening, and primary resistance, that manifests itself in patients who didn't received any previous treatment. In this case, infection has probably been caused by *bacilli* originary from a patient with secondary resistance[3] .

Early observations about cases of resistance were raised in the late 40's[4] . At this time, sulfonamide drugs as promin, diasone and DDS (4,4'-diaminodiphenylsulfone), were already used to treat the disease.

DDS was the first drug experimentally proven to induce bacillary resistance. That was possible only after Shepard standardized the footpad inoculation of isogenic mice, in 1960[5]. Following this method, Petit *et al*[6]. were able to prove experimentally, in 1964, the first cases of *bacilli* resistance to DDS. When we discuss resistance, this is the most important anti-leprosy drug because it was largely used as monotherapy, for more than 30 years. The pattern of resistance is classified as partial (low), intermediary or total (high), according to the *bacilli's* ability to reproduce in mouse treated with 0,0001g%, 0,001g%

or 0,01g% of DDS, respectively, in their feed. The concentration of 0,01g% corresponds to a dose of 100 mg to human beings[3].

Many years later, even with the introduction of MDT, several reports of primary and secondary resistance, not only to DDS but also to RMP, clofazimine (CLO) and ofloxacin (OFLO), were described in various countries[7-12].

The Instituto Lauro de Souza Lima has been doing experimental studies using the Shepard's mouse foot pad technique, since 1986. Analyzing 30 lepromatous patients from Bauru (SP), who constituted a risk group to sulfone resistance, Costa *et al.*[13] obtained a 2,86% resistance rate to DDS. Although significant, this figure does not express our epidemiologic reality, probably because the techniques for resistant *bacilli* detection - mouse footpad inoculation and molecular biology - are expensive and not available in most of laboratories.

There are important criteria to suspect resistance, such as relapses in multibacillary patients already treated or undergoing treatment, or unsatisfactory clinical response that calls the clinician's attention. The aim of our study was to detect DDS and RMP resistant strains among treated patients with clinical signs of relapse. These individuals came from various cities of the São Paulo State. In this study we used Shepard´s mouse footpad technique.

Patients and Methods

Patients: from 1994 to 2002, we evaluated 40 treated patients (n = 40) of both sexes, clinically diagnosed with lepromatous (30) or borderline lepromatous (10) leprosy. All patients had bacilloscopic index (BI) > 3+, with clinical suspicion of relapse. Only 29 patients had their diagnosis confirmed by histopathology. In 22 cases, skin biopsy showed the disease in activity; in one case with type 2 reaction and in another case, with reversal reaction. There were also five cases of regression. The patients were from São Paulo State and capital. They were referred to the ILSL by the SUS public health system. Nine cases were seen at the Divisão de Hansenologia e Dermatologia Sanitária/SP, and 31 at the ILSL. All of these patients reported having undergone some kind of treatment (promin, diasone, dapsone, MDT-MB/ WHO); two patients could not inform which medication they had used. At clinical dermatological evaluation, none of these patients were receiving treatment. The study included only those patients whose treatment had been concluded at least five years before, and who showed new lesions and positive BI[14]. They were all examined by a dermatologist for clinical evaluation, biopsy collection and bacilloscopic examination.

Biopsy: two fragments of lesion were collected. One was sent to histopathological examination, and the other was inoculated in a mouse's left footpad to test susceptibility to drugs. Biopsy processing for inoculation: *bacilli's* DDS and RMP susceptibility tests were done according to Shepard's technique[15]. The biopsy was minced in a tissue homogenizer containing 2 ml of Hanks' balanced salt solution (HBSS Gibco BRL®), to obtain the bacilli suspension. Afterwards, an amount of 30 ml of the suspension was laid on microscopy slides, fixed and stained with the Ziehl-Neelsen staining. All the procedures were carried out in antiseptic conditions, in laminar flow chamber.

Inoculation: 40 young BALB/c mice of both sexes were inoculated by intradermal injection in the left hind footpad, with 104 *bacilli*/0,03 ml. The animals were divided in five groups: control (diet without drugs), DDS 0,01g%, DDS 0,001g%, DDS 0,0001g% and RMF 10 mg/ kg. DDS (Sigma®) was added to daily feed and RMF (Merck®) was administrated by gavage, once a week, for six months. The animals were kept in controlled environment, with an average temperature of 22ºC, receiving water and feed *ad libitum*.

Period of inoculation: the animals were anaesthetized and sacrificed by cervical dislocation ten months after inoculation; the footpad tissue was excised and processed following the protocol in use for the patient's biopsy, with subsequent counting of *bacilli*.

Results evaluation: the index of =105 *bacilli*/footpad[16] was considered as significant bacillary multiplication. The results were interpreted as sensitive only when there was bacillary multiplication in the control group; resistant, when the multiplication occurred in the control group and also in any animal which had received DDS or RMP; inconclusive, whenever there wasn't bacillary multiplication among the animals of the control group and the treated animals. The interpretation of results for the cases of DDS resistance is exposed in Chart 1.

Chart 1. Results interpretation of *Mycobacterium leprae's* resistance to dapsone

Bacillary multiplication (diet with DDS)			Types of resistance
0,01g%	0,001g%	0,0001g%	
- [a]	-	-	Total resistance
- [b]	+	+	Intermediary resistance
-	-	+	Partial resistance

≥ 105 bacilli/ footpad =< 105 bacilli/ footpad

Results

Between December 1994 and May 2002, we investigated 40 cases with clinical suspicion of relapse, which constituted a risk group for drug resistance. Among 40 investigated cases, 22 (55%) showed inconclusive results, i.e., there was no bacillary multiplication (105 *bacilli*/foot pad), in the control group; five cases (12,5%) presented *bacilli* sensitive to DDS and RMF, with bacillary multiplication only in the control group (105 *bacilli*/foot pad). Resistance to DDS was observed in 11 cases (27,5%) five cases of total resistance (45,4%), one of intermediary resistance (9,1%) and five of partial resistance (45,4%). We did not observe any case of multiple resistance (Table 1).

Table 1. Results of test of susceptibility to DDS and RFP, achieved by mouse footpad inoculation among 40 leprosy patients from São Paulo State, with clinical suspicion of relapse.

Susceptibility to drugs (n 40)					
		Resistant			
		Dapsone			Rifampin
Sensitive [a]	Inconclusive [b]	0,01g%	0,001g%	0,0001g%	10mg/kg
05	22	05	01	05	02

[a] Bacillary multiplication only in control group.
[b] Absence of bacillary multiplication in control group.

The profile of patients who had DDS or RMP resistant *bacilli* is described in Table 2. This information was obtained from their medical files.

Table 2. Profile of patients with clinical suspicion of relapse, showing dapsone or rifampin resistant *bacilli* in mouse footpad.

Nº	Sex	Clinical form	First manifestation of disease (year)[a]	Period of evolution year)[b]	Therapy	Resistance
1	F	L	1947	51	PR, DDS	DDS P
2	F	I.	1945	53	DI	DDS – P
3	M	L	NR	15*	NR	DDS P
4	M	L	1969	32	DDS	DDS – T
5	M	I.	1943	59	DDS, MDT/R	DDS – T
6	M	L	1972	23	NR	DDS T
7	F	I.	1941	54	NR	DDS – P
8	M	L	1947	48	PR, DI, DDS/ IR	DDS T
9	F	L	NR	7*	NR	DDS P
10	F	L	1954	42	PR, DDS	DDS T
11	M	I.	1947	50	PR, DI, MDT/ IR	DDS – I
12	M	I.	1979	21	MDT/ IR	RMP

[a] As reported by patient; b period of the disease's evolution till relapse; *approximate period
NR = not reported, F = female, M = male, L= lepromatous, T= total, P= partial, I= intermediary, PR= promin, DI= diasone, R= regular, IR= irregular.

Some of this data - such as treatment - were not very clear, because most of the patients were referred to treatment at the Instituto Lauro de Souza Lima by other health unities. Furthermore, in some cases, the patient himself could not accurately inform the name of the medication(s) he had taken. Only cases 5, 11 and 12 had already utilized the MDT/MB/24 - WHO scheme. Among these three patients, only one was regularly medicated; the two others used the scheme irregularly. The other subjects of the study utilized promin, diasone and DDS. Cases 1, 2, 3, 4, 5, 7, 8 and 11 had histopathological diagnosis of active leprosy. The amount of time between the first manifestation of the disease (according to the patient) and the relapse varied from 7 to 59 years. All these patients had been discharged as cured.

Conclusions

The detection of resistant *bacilli* in patients that do not show clinical recovery after treinament is an important question to be considered in the prevention of news cases of resistance, mainly primary resistence.

Besides, a multibacilar, patient with active disease even after treatment, can become infection source again. In spite of no case of multiple resistant have been ocurred, this possibily should be considerated, since there is previously related cases.The emergency of resistant strains to RFP, as well their dissemination, can represent difficults to treatement and constitutes a serious risk to the leprosy control programs. The experimental confirmation of suspicious of cases of resistant is also important to help the choice alternative treatment and to take off the possibility of persistant *bacilli*.

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Resumo

Os relatos de resistência a dapsona e rifampicina fizeram com que a Organização Mundial de Saúde preconizasse, em 1981, a poliquimioterapia para o tratamento da hanseníase. A prevenção da seleção de cepas mutantes resistentes às drogas é um de seus principais objetivos. A dapsona foi a primeira droga a ter comprovação experimental de resistência e isto só foi possível depois que a técnica de inoculação do bacilo em coxim plantar de camundongos foi padronizada em 1960. Critérios importantes a serem considerados para se suspeitar de resistência seriam recidivas em pacientes multibacilares já tratados, ou em tratamento,

ou resposta clínica insatisfatória. Nosso estudo teve por objetivo detectar cepas resistentes à dapsona e rifampicina entre 40 pacientes tratados, com sinais clínicos de recidiva, procedentes de cidades do Estado de São Paulo e capital, utilizando a técnica de inoculação do bacilo em coxim plantar de camundongos. Foram observados bacilos resistentes à dapsona em 11 casos, sendo 05 de resistência total, 01 intermediária e 05 parcial. Bacilos resistentes à rifampicina foram observados em apenas 02 casos. Não se observou nenhum caso de resistência múltipla. O alto índice obtido de resistência à dapsona, provavelmente é decorrência de muitos anos de monoterapia sulfônica ou de seus derivados. No caso da rifampicina, provavelmente a droga foi utilizada de forma irregular, em monoterapia ou ainda, o paciente pode ter utilizado-a previamente para tratar outra moléstia. A detecção de bacilos resistentes entre pacientes que não melhoram clinicamente ou que recidivam após o tratamento, é uma questão importante a ser considerada na prevenção futura de novos casos de resistência. A emergência de cepas resistentes, especialmente à rifampicina, bem como a sua disseminação, pode trazer dificuldades ao tratamento do paciente e se constituir em ameaça aos programas de controle da hanseníase.

Palavras-chave: *Mycobacterium leprae*; resistência; dapsona; rifampicina

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