Early Detection of Rifampin in Human Nerve Tissue after an Oral Dose of 600 Milligrams

M. GUEBRE-XABIER,1,2 E. J. SHANNON,3* R. KAazen,2 Z. KEBRET,2 and D. FROMMEL1

Armauer Hansen Research Institute1 and All Africa Leprosy Rehabilitation and Training Center,2 Addis Ababa, Ethiopia, and Gillis W. Long Hansen’s Disease Center at Louisiana State University, Baton Rouge, Louisiana3

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Rifampin in picogram quantities inhibited the ability of Mycobacterium bovis 44 BCG P3 to release 14CO2 from the oxidation of [14C]palmitic acid. By using these mycobacteria in a bioassay, samples of serum and posterior tibial nerve were assayed for inhibitory concentrations of rifampin. Within 8 to 12 h after ingestion of 600 mg of rifampin, the drug was detected in eight patients in concentrations ranging from 0.52 to 4.1 μg/ml in serum and in concentrations ranging from 0.6 to 6.3 ng/mg in posterior tibial nerve fiber tissue.

Introduction. Mycobacterium leprae has a tropism for nerves, where it is assumed to persist despite years of monotherapy with dapson. Recently, in multiple-drug-treatment (MDT) trials conducted in Bamako, Nigeria, and Chingleput, India, it was concluded that viable drug-susceptible M. leprae could be present in about 10% of multibacillary patients and that these bacteria were not killed by any of the combinations of dapson, rifampin, and clofazimine employed in the MDT trials (4). These observations favor a hypothesis of poor delivery of these antileprosy drugs into human nerve tissue.

Following intravenous injection of rifampin into a primate (7) and after administration of multiple oral doses of rifampin, dapson, isoniazid, and pyrazamide to sheep and dogs (2), the drugs were detected in peripheral nerves. From these animal models, it was speculated that the finding could be extended to humans, and it was theorized that M. leprae could persist in tissues by being physiologically dormant and thereby escaping the action of drugs (7).

The present investigation with humans supports the finding from the animal studies about delivery and penetration of rifampin into nerve tissue.

Materials and Methods. (i) Development of the bioassay. Mycobacterium bovis 44 BCG P3 was grown in 7H9 Middlebrook broth that had been supplemented with 10% (vol/vol) albumin-dextrose-catalase (ADC) (Difco, Detroit, Mich.). BCG cells at 10⁶/ml of 7H9-ADC medium were incubated with rifampin at concentrations ranging from 2.0 to 2.0 × 10⁻⁶ mg/ml of culture medium. The BCG cultures were enclosed in sterile Nunc tubes (Nunc, Roskilde, Denmark) with their caps tightened. On the fourth day, 0.5 μCi of [1-14C]palmitic acid (59 mCi/mmol; Amersham International, Little Chalfont, Amersham, Buckinghamshire, United Kingdom) was added to each culture. The Nunc tubes, with their caps loosened, were placed in a modified Buddeeyer-type incubation vessel (8). Next to the cultures of BCG was a sealed glass vessel (15 by 25 mm) containing a dried strip of Whatman DE42 filter paper (Whatman, Inc., Clifton, N.J.). The paper had been previously dipped into concentrated liquid scintillation counting solution (Liquifluor PPO-PPOP [2,5-diphenyloxazole-1,4-bis[5-phenyloxazole]benzene]) toluene concentrate (New England Nuclear, Boston, Mass.) with 1% (vol/vol) Triton X-100 and an equal volume of 4 N NaOH. The 14CO2 captured on the filter paper was measured daily by using a RackBeta Liquid Scintillation Counter (LKB, Pharmacia, Uppsala, Sweden).

(ii) Patients. Samples of blood and nerve tissue were taken from patients ranging from 17 to 70 years of age. The patients participating in experiments I and II were multidrug-treated, multibacillary leprosy patients with inactive disease. They had been released from treatment for at least 1 year prior to their surgery. In experiment III, patient T (Pt. T) was not a leprosy patient and had never received any antileprosy drugs. Pt. T, classification unknown, was a “burnt-out” patient who had not received any recorded antileprosy treatment. Pt. A was treated as a multibacillary leprosy patient and had completed MDT 9 years prior to her amputation. All the patients were attending the All Africa Leprosy Rehabilitation and Training (ALERT) clinics for a surgical amputation procedure because of untreatable ulcers and noncorrectable deformities.

After informed consent had been given, blood was drawn from each patient and then an oral dose of 600 mg of rifampin was administered. Approximately 8 to 12 h later, a second blood sample was taken. Following spinal anesthesia, the patients underwent the surgical amputation procedure. The posterior tibial nerve was removed from the amputated leg from the medical malleolus and 10 cm proximally under sterile conditions. The nerve and serum samples were frozen at −79°C. When the nerve and serum samples from two or more patients were collected, they were bioassayed. On three separate occasions, the serum and nerve samples were assayed for rifampin and the bioassays were designated experiment I, experiment II, and experiment III.

(iii) Bioassay for rifampin in the blood. To utilize the bioassay to estimate the concentration of rifampin in patient serum, a reference standard curve was prepared as follows: three replicate cultures contained 10⁷ BCG cells in 1.0 ml of 7H9-ADC medium with 10 μl of normal serum from a healthy staff member and 5 μl of methanol, and three replicate cultures of 1.0 ml of 7H9-ADC medium with 10⁷ BCG cells contained 10 μl of normal serum with 5 μl of methanol with rifampin (Sigma, St. Louis, Mo.) dissolved to effect rifampin concentrations of 0.02, 0.04, and 2.0 μg/ml. To determine if rifampin was present in the patient serum, three replicate cultures of 10⁴ BCG cells in 1.0 ml of 7H9-ADC medium were incubated with 10 μl of methanol and 10 μl of serum taken prior to the ingestion of rifampin; alternatively, the BCG-methanol cul-
tudes were incubated with 10 μl of serum drawn after ingestion of rifampin. All the cultures were enclosed in sterile Nunc tubes with the caps tightened. The cultures were incubated for 2 days at 37°C in a high-humidity incubator. On the third day, 0.5 μCi of [14C]palmitic acid was added to each culture. The Nunc tubes, with loosened caps, were placed into the modified Buddemeyer-type incubation vessel. The 14CO2 captured on the filter paper was measured daily.

(iv) Bioassay for rifampin in nerve tissue. As normal serum had been added to the BCG cultures as a control in the bioassay for rifampin in the blood, a similar control in the nerve study used a section of the posterior tibial nerve from a decreased nonleprosy–non-MDT individual (normal nerve). Approximately 2-cm-long portions of the normal nerve and the patient’s nerve tissue were processed for the extraction of rifampin. The epineurium was removed, and the endo- and perineurial zones were processed for extraction of rifampin. The nerve fibers were suspended in 5.0 ml of sterile 0.85% saline and ground with a pestle in a mortar. In experiment I and experiment II, an aliquot of this suspension was bioassayed. In experiment III, methanol was used to facilitate extraction of rifampin. In this procedure, 5 ml of methanol was

added to the 5.0 ml of ground suspension of nerve. The methanol-saline-nerve suspension was centrifuged at 250 × g for 5 min. The methanol fraction was lyophilized, weighed, and reconstituted in 2.0 ml of 7H9-ADC medium.

To estimate the concentration of rifampin in the processed patient nerve fiber tissue, a standard reference curve using BCG cultures was prepared. One set of cultures contained a 100-μl volume from the sham-rifampin-extracted normal nerve suspension and 10⁴ BCG cells in 1.0 ml of 7H9-ADC medium with 10 μl of methanol; a second set of cultures contained 10⁴ BCG cells in 1.0 ml of 7H9-ADC medium and 100 μl from the sham-rifampin-extracted normal nerve suspension with 10 μl of a solution containing various concentrations of rifampin in methanol. The final concentration of rifampin was 0.20, 0.02, or 0.002 μg/ml.

One hundred microliters of the patient nerve tissue suspension, which had been processed for rifampin extraction, was added to the 1.0-ml cultures containing 10⁴ BCG cells and 5 μl of methanol. The cultures were incubated and monitored for the evolution of 14CO₂ as described for the bioassay for rifampin in serum.

Results. After exposure of BCG cells to concentrations of

FIG. 1. BCG cells, 10⁴ bacilli per culture, were incubated at 37°C with rifampin at concentrations ranging from 2.0 to 2.0 × 10⁻⁶ mg/ml of culture. After 4 days, 0.5 μCi of [14C]palmitic acid was added to each culture. The amount of 14CO₂ evolved from the oxidation of radiolabelled palmitic acid was monitored daily for 5 days by using a modified Buddemeyer system. All concentrations of rifampin were significantly inhibitory after 2 days of culture, with a P value of <0.01, according to Dunnett’s t test. The cultures incubated with the lowest concentration of rifampin, 2.0 ng/ml, showed inhibition of oxidative metabolism of BCG on the fourth and fifth days of incubation (Dunnett’s t test, P < 0.0001). Error bars indicate standard deviations.
rifampin ranging from 2.0 to 2.0 × 10^{-6} mg/ml, their ability to oxidize [14C]palmitic acid and release 14CO2 was significantly inhibited. In the cultures of BCG incubated with 2.0 ng/ml, the lowest concentration of rifampin tested, there was inhibition of oxidative metabolism on the fourth and fifth days of incubation (Fig. 1).

By using this bioassay, the concentrations of rifampin in the sera of patients receiving rifampin were estimated. On the basis of the 14CO2 counts per minute of 8-day cultures of BCG receiving 10 μl of normal serum (the mean cpm ranged from 64,010 to 11,795) and those receiving 10 μl of serum from eight patients prior to their ingestion of rifampin (the mean cpm ranged from 87,000 to 17,684), there was no indication of the presence of rifampin in the patient serum. However, comparing the range of 87,000 to 17,684 mean cpm obtained with serum collected prior to the ingestion of rifampin with the values obtained with serum collected 8 to 12 h after the ingestion of rifampin (the mean cpm ranged from 84 to 8,454) revealed a significant reduction in counts per minute. The difference in counts per minute before and after ingestion of rifampin among a group of three subjects bioassayed in experiment III is illustrated in Fig. 2.

A summary of the estimates of the concentrations of rifampin in the sera of eight patients is given in Table 1. For each experiment, a standard curve for linear regression analysis was prepared by using the log_{10} transformation of the individual counts per minute from each of three control cultures of normal serum with and without rifampin as the independent vari-

### Table 1. Concentrations of rifampin in serum and posterior tibial nerve tissue 8 to 12 h after ingestion of 600 mg of rifampin

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Patient</th>
<th>Serum (μg/ml)</th>
<th>Nerve tissue (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>0.510</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.519</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00</td>
<td>0.6</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>0.984</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.952</td>
<td>4.8</td>
</tr>
<tr>
<td>III</td>
<td>T</td>
<td>4.18</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.14</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>3.49</td>
<td>6.2</td>
</tr>
</tbody>
</table>

**a** \( \tau = 1.59 \pm 1.4 \mu g/ml \)

**b** \( \tau = 4.2 \pm 2.1 \text{ ng/mg} \)
able \( (x) \). The mean \( \log_{10} \) counts per minute of the patient serum was used as the dependent variable \( (y) \), and the quantity of rifampin was then calculated by using the equation for the linear regression.

To determine if rifampin was present in the patient nerve fiber tissue, three separate experiments with three separate standard curves were analyzed. A summary of the estimated concentrations of rifampin in the nerves of eight patients is given in Table 1.

Figure 3 illustrates the evolution of \(^{14}\)CO\(_2\) in BCG cultures treated with normal nerve tissue with and without addition of rifampin and with nerve tissue from three patients from experiment III. The mean counts per minute of the methanol extract of the nerves from these patients was significantly less than the mean counts per minute of the methanol extract of normal nerve cultures which received no rifampin (Fig. 3, line A) and was also less than the value for methanol extracts of normal nerve tissue with rifampin added at 2.0 ng/ml (line B). Table 2 summarizes the analysis of the data used to estimate the concentrations of rifampin in the methanol extracts of nerve tissues from Pt. A, Pt. F, and Pt. T.

**Discussion.** This study demonstrated that rifampin was present in serum and penetrated into the posterior tibial nerve fiber tissue of humans. None of the patients had detectable levels of rifampin in serum samples taken prior to ingestion of rifampin, whereas all the patients had rifampin in their sera after the single 600-mg oral dose. The amount of rifampin estimated to be present in the serum is comparable to that estimated from previous pharmacokinetic studies with humans (1). From previous studies, depending on the frequency with which rifampin was received and the dose of rifampin, the peak concentrations varied among subjects and also within subjects. In general, approximately 2 h after a 600-mg oral dose of rifampin a peak serum drug concentration of approximately 10

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**TABLE 2. Estimation of rifampin content in nerve tissue**

<table>
<thead>
<tr>
<th>Rifampin concn in standard curve bioassay ( (\mu g/ml) ) or nerve tissue sample (patient)</th>
<th>(^{14})CO(_2) cpm for 8-day cultures ( (\bar{x} \pm SD) \ (n)^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>33,867 ± 3,195 (4)</td>
</tr>
<tr>
<td>0.002</td>
<td>7,654 ± 2,217 (4)</td>
</tr>
<tr>
<td>0.02</td>
<td>340 ± 22 (4)</td>
</tr>
<tr>
<td>0.2</td>
<td>173 ± 26 (4)</td>
</tr>
<tr>
<td>T</td>
<td>1,094 ± 68 (3) [0.09 ( \mu g/ml )]</td>
</tr>
<tr>
<td>F</td>
<td>1,237 ± 244 (3) [0.08 ( \mu g/ml )]</td>
</tr>
<tr>
<td>A</td>
<td>1,717 ± 5,128 (3) [0.10 ( \mu g/ml )]</td>
</tr>
</tbody>
</table>

* Concentration of rifampin (in micrograms per milliliter of BCG culture) with normal nerve tissue.

* For the standard curve, \( r = 0.714 \) and \( P = 0.0019 \) (value for equation of the line after log transformation of counts per minute from the standard curve).

\( \text{Log}_{10} y = 3.24 + -7.76 \) (log10 x).

* Values in square brackets are predicted amounts of rifampin in patient nerve tissue cultured with BCG.
µg/ml was detected, and the biologic half-life (t₁/₂) of rifampin has been estimated to be 3 h. On the basis of these estimates (a serum rifampin concentration of 10 µg/ml [C₀] after 2 h and a t₁/₂ of 3.0 h [k = 0.693/t₁/₂]), the estimated concentration (C) could have been 0.99 µg/ml at a time (t) of 10 h (12 – 2 h) and 2.5 µg/ml at t = 6 h (8 – 2 h) (C = C₀e⁻kt), (9). This range of calculated values for rifampin in serum is compatible with those obtained in this study.

The contribution of rifampin contained in blood remaining in the processed nerves is difficult to estimate. McDougall et al. (7) prepared 4- to 5-cm-long segments of the right and left sciatic nerves of a monkey for extraction of rifampin, similar to the way nerves were prepared in the present study. On the basis of data from the human medulla, McDougall et al. estimate that the maximum contribution of [¹⁴C]rifampin in blood in nerve tissue could be 20 ng/10⁵ mg. Therefore, from this estimate, the mean concentration of rifampin in nerve tissue of 4.2 ng/mg (Table 1) could be reduced by only 0.2 µg/mg. Six hours after the animal had been given an intravenous dose of 6.48 mg of [¹⁴C]rifampin per kg of body weight, McDougall et al. (7) detected 2.09 µg of [¹⁴C]rifampin per g of sciatic nerve tissue in a rhesus monkey. Allen et al., in 1975 (2), showed that following multiple oral doses of rifampin, rifampin was present at 4.4 µg/g (4.4 ng/mg) in the sciatic nerve tissue of a dog and at 1.5 µg/g in the sciatic nerve tissue of a sheep. The nanogram-per-milligram concentrations of rifampin in human nerve tissue (Table 1) are compatible with those found in these animal studies.

Pt. T and F had received rifampin only once on the occasion of the experimental protocol; the other six patients had been enrolled in the long-term MDT program recommended by the World Health Organization. These patients (four male and two female, ranging in age from 17 to 70 years) had received 600 mg of rifampin and 300 mg of clofazimine monthly under supervision and presumably 100 mg of dapsone and 50 mg of clofazimine self-administered daily. Despite the fact that the patients had not received MDT for at least a year, there is the possibility of residual antimycobacterial drugs, especially clofazimine, in the nerve tissue. On the basis of an average biological t₁/₂ of 28 h for dapsone (3) and 3 h for rifampin (1, 3), it is highly unlikely that there was any carryover of these drugs in the bioassay. Mathematically, however, 1 year after release from MDT the patients could still have clofazimine in their nerve tissues. Clofazimine was detected at 1.7 µg/g of nerve tissue in a patient who had a total intake of 19.9 g of drug for some 243 days until his death (6). On the basis of these data and assuming the deposition of clofazimine in nerve tissue is cumulative without decay and directly related to the total intake of 43.7 g as prescribed for MDT, the patients could, at an optimistic estimate, have 3.7 mg of drug present per g of nerve tissue at the end of their MDT regimen. If 70 days is the minimum estimated t₁/₂ of clofazimine in skin tissue of humans (5), 1 year after release from MDT (estimated minimal release time for patients 1, 2, 3, 4, and 5), patients could have clofazimine present at 0.115 mg/g of nerve tissue. Patient A, who was released from MDT as a multibacillary leprosy patient 9 years prior to her surgery, mathematically could have clofazimine present in her nerve tissue at 2.78 × 10⁻¹⁴ mg/g. In a similar radiorespirometric assay, three replicate cultures of this strain of BCG in the absence of clofazimine gave 12,201 ± 5,788 cpm, while three replicate cultures with clofazimine at 20.0 ng/ml gave 10,954 ± 1,315 cpm, three replicate cultures with clofazimine at 2.0 µg/ml gave 11,074 ± 512 cpm, and three replicate cultures with clofazimine at 0.2 µg/ml gave 10,851 ± 1,654 cpm (2a).

Despite the experimental evidence for adequate penetration of the anti- M. leprae drugs currently used in MDT, the presence in nerve tissue of persistent M. leprae strains, especially those that may be genetically resistant to one or more of the drugs currently used in MDT, warrants the continued search for new antileprosy drugs.

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