Isolation of Three Mycobacterium ulcerans Strains Resistant to Rifampin after Experimental Chemotherapy of Mice

Laurent Marsollier,1* Nadine Honoré,2 Pierre Legras,3 Anne Lise Manceau,4 Henri Kouakou,4 Bernard Carbonnelle,1 and Stewart T. Cole2

Laboratoire de Bactériologie-Virologie-Hygienè Hospitalière, CHU, 49033 Angers Cedex 01,1 Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, 75724 Paris Cedex 15,2 and Animalerie Hospitalo-Universitaire, Faculté de Médecine, 49000 Angers,3 France, and Institut Raoul Follereau, Adzopé, Côte d’Ivoire4

Received 17 May 2002/Returned for modification 26 July 2002/Accepted 23 December 2002

MATERIALS AND METHODS

Mycobacterial strains and isolation of rifampin-resistant mutants. M. ulcerans (strain 01G897) was originally isolated from a skin biopsy specimen of a human patient from French Guyana (5) and was passaged on Löwenstein-Jensen medium at 30°C (Sanofi Diagnostics Pasteur, Marnes la Coquette, France). In the first experiment, 30 μl of a suspension containing 5 × 10⁶ cells was injected subcutaneously into a footpad of each of 120 BALB/c mice (age, 5 weeks; Iffa Credo, Saint Germain sur l’Arbresle, France). After inoculation, the initial suspension was frozen at −80°C. Five weeks later, all the mice showed inflammatory lesions. The mice were then dispatched at random into two groups. The mice in group 1 (50 mice) were not treated and was used as the control group. Five mice were killed every week, and the number of bacilli present in infected tissues was counted. The mice in group 2 (70 mice) were treated with rifampin (10 mg/kg of body weight 5 days a week). Five mice were killed every 2 weeks for 3 months, and the number of bacilli present in infected tissues was counted. At the end of the 90-day treatment, 30 mice from group 2 were kept for a further 3 months for clinical observations to detect the emergence of any possible resistant strains.

In the second experiment, the initial suspension (containing 1.7 × 10⁶ bacilli per ml) was inoculated on Löwenstein-Jensen medium with or without rifampin at 30 μg/ml, as described for the proportion method (2). This suspension was also injected subcutaneously into a footpad of each of 40 BALB/c mice. The mice were treated with rifampin for 3 months by the same method used in the first experiment.

Enumeration of M. ulcerans in infected tissues. The tissue specimens from five mice were weighed, minced with disposable scalpels in a petri dish, and ground with a Potter-Elvehjem homogenizer (size 22; Kimble/Kontes, Vineland, N.J.) in 0.15 M NaCl to obtain a 10-fold dilution. Smears of suspensions were stained by the Zielh-Neelsen procedure. The suspension was decontaminated with an equal volume of N-acetyl-L-cysteine sodium hydroxide (2%) as described previously (11), and cultures were obtained by inoculating 0.2 ml onto two Löwenstein-Jensen slants (Sanofi Diagnostics Pasteur). After Zielh-Neelsen staining, the acid-fast bacilli were counted by the method of Shepard and Rae (19).

Study of efficiency of rifampin. After proliferation by the standard method (22), the activity of rifampin was tested against four strains of M. ulcerans: wild-type strain 897 and three rifampin-resistant strains (strains R₁, R₂, and R₃) from mice that had relapses after 90 days of treatment. The precise level of resistance to rifampin was determined by measuring the MIC in liquid medium by the method described by Rastogi et al. (18). In our experiments, the MIC corresponded to the lowest amount of antibiotic that inhibited 99% of the growth of M. ulcerans.

Proof of infectious activities of resistant strains and demonstration of acquired resistance. After culture on Löwenstein-Jensen medium, bacilli of strains 897, R₁, and R₃ were suspended in saline, centrifuged, and resuspended in saline.
to obtain $5 \times 10^9$ bacilli in 30 μl. Three groups of 20 BALB/c mice each were constituted: the wild-type strain was injected into the mice in group 1, while the mice in groups 2 and 3 received bacilli from strains R1 and R2, respectively. Approximately 4 weeks later, edema appeared on the legs of all the mice. Ten mice from each group were treated with rifampin for 3 months, and clinical observations were made each week.

**Sequencing of the rpoB gene.** After 6 weeks of culture on Löwenstein-Jensen medium, the colonies were harvested and a suspension of the bacteria was made in 200 μl of sterile distilled water. The bacteria were heated at 100°C for 30 min and then lysed by five cycles of boiling and freezing (25) before a second treatment at 100°C for 10 min. A fragment of 326 bp of rpoB was amplified by PCR with *M. ulcerans*–specific primers MulrpoB-R (5’-TGCGCACGTTGAGT GAGC-3’) and MulrpoB-F (5’-GACGTGCACCCCGTCGCACT-3’) (12). The reaction was carried out in a final volume of 50 μl containing 5 μl of DNA, 5 μl of 10× PCR buffer [170 mM (NH4)2SO4, 600 mM Tris-HCl (pH 8.8), 20 mM MgCl2, 100 mM β-mercaptoethanol], 5 μl of dimethyl sulfoxide, 5 μl of deoxynucleoside triphosphates (5 mM each), 5 μl of primers (2 μM), 25 μl of distilled water, and 2 U of Taq polymerase (Gibco BRL). The amplification was performed by 45 cycles of 1 min at 92°C, 2 min at 59°C, and 2 min at 72°C, with a final step at 72°C for 10 min. After purification the amplified fragments were sequenced with an ABI 377 automated DNA sequencer (Applied Biosystems), BigDye terminators, and primers MulrpoB-R and MulrpoB-F.

**RESULTS**

**Effect of rifampin on experimentally infected mice.** At the beginning of the treatment, which corresponds to 5 weeks after inoculation, the number of visible acid-fast bacilli counted in infected tissues was $3.24 \times 10^7$ per g of tissue. Among these bacilli, $4.1 \times 10^6$ organisms per g of tissue were viable. At this stage the feet of the mice displayed extensive edema that had spread over the leg. In the control group, the number of organisms increased until the 9th week after inoculation, with $10^9$ viable bacilli per g of tissue being attained. In the group treated with rifampin, the number of bacilli increased for the first 15 days (7th week after inoculation), reaching a maximum level of $5.6 \times 10^6$ viable bacilli per g of tissue. Subsequently, the number of viable organisms decreased, and from weeks 12 to 16, the end of the treatment, no viable bacilli were found. Inflammatory reactions disappeared, although some redness remained on the footpads. After 90 days of treatment with rifampin, 30 mice were alive and were kept for observation. At 6 and 7 weeks after the end of the treatment, relapses occurred in two mice, which developed a large inflammation of the footpads. Tissue samples were then taken from these lesions, and culture of the tissue led to the isolation of *M. ulcerans*. No relapses occurred in the 28 mice surviving at the end of these 3 months.

In the second experiment, 40 mice were kept alive after 3 months of treatment. During the 3 months after the end of treatment one mouse had a relapse, and *M. ulcerans* was isolated from the footpad tissues.

**Rifampin sensitivities of strains.** *M. ulcerans* 897 was inhibited by 1 μg of rifampin per ml, whereas no inhibition was observed for the three strains isolated from the mice in which relapses occurred in the two experiments. The MIC, determined by a dilution method, was 0.5 μg/ml for the wild-type strain, whereas the MICs were 8 μg/ml for strains R1 and R2 and 32 μg/ml for strain R3, these correspond to >8-fold and >16-fold increases, respectively.

**Detection of rifampin-resistant mutants in a bacterial inoculum.** No rifampin-resistant mutants were isolated from the suspension from which samples were taken for inoculation into the mice, indicating that the number of spontaneously resistant mutants in the initial inoculum was below the limit of detection of this method.

**Comparison of pathological effects of strains R1 and R2 and the wild-type strain.** Whether the strain was sensitive or resistant to rifampin, the clinical evolutions of the infections were similar in the untreated mice. Five weeks after inoculation, all of the mice had large areas of inflammatory edema over their feet. There were no ulcerations. Nine weeks after inoculation, the whole leg of each mouse showed major edema and extensive lesions. Between the 9th and the 12th weeks, all of the animals died of secondary infections.

Mice infected with *M. ulcerans* 897 and treated with rifampin showed inflammatory lesions that decreased after 30 days of treatment but an edema that persisted on the footpads. No inflammation was observed 60 days after the beginning of the treatment (12 weeks after inoculation); only redness of the footpad was observed.

Similar clinical observations were made for animals (20 mice) inoculated with strains resistant to rifampin. After 30 days of treatment, 15 of 20 mice showed major edema of the leg, similar to that observed in the untreated mice. Five mice had an edema of the footpad which decreased.

After 90 days of treatment, 15 mice had died. The MICs for the bacilli isolated from infected tissues were the same as those for strains R1 and R2, that is, 8 μg/ml. Among the five surviving mice, one mouse had edema on the whole leg. *M. ulcerans* was isolated from tissues taken from this mouse, and the MIC for that isolate was the same as that for strains R1 and R2. No bacilli were isolated from the other four mice.

With respect to mutant R3, a preliminary study showed that the strain is pathogenic for mice and that the rifampin treatment was inefficient.

**Sequencing of rpoB of *M. ulcerans*.** The 326-bp amplicon generated by PCR was sequenced. The sequence of this region showed 90.2% identity with the *M. leprae* sequence (9). Figures 1 and 2 show the alignment of a 62-bp fragment, corresponding to the rifampin resistance-determining region, from both organisms. When the sequence of the same region of the rpoB gene from resistant strains R1 and R2, was determined, a missense mutation was found in codon 416 (TCC→TTC), resulting in the replacement of a serine by a phenylalanine (Fig. 1 and 2). The same point mutation was observed in both strain R1 and strain R2. For strain R3, a different missense mutation was found in codon 420 (CAC→TAC), resulting in the replacement of a histidine by a tyrosine (Fig. 2).

**DISCUSSION**

Ansamycins (rifamycin and rifampin) were discovered in 1967 and have been used for therapy since 1970. Their efficacies as treatments for tuberculosis have been proved. In 1981, rifampin was included as part of the treatment regimen for leprosy and became one of the main drugs used in multidrug therapy (26). Following an epidemic of Buruli ulcer in Uganda in the 1960s (23), the activity of rifampin as a treatment for that disease was tested in mice experimentally infected with *M. ulcerans* (8, 21). The observations from our study, which was prompted by the epidemic occurring in West Africa (1), mirror
what Dega et al. (4) observed. The efficiency of the treatment has been confirmed in studies with experimentally infected mice. Rifampin had a high level of activity when it was used to treat mice experimentally infected with $10^6$ bacilli. By use of such an inoculum, the mice showed large edemas of the foot-pads after 5 weeks, when treatment with rifampin began. Thus, we concluded that, at the beginning of the appearance of ulcerative lesions, the infection in mice is very similar to the one observed in humans, when people become concerned and obtain medical advice. No viable bacilli were found 4 weeks

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![FIG. 2. Nucleotide sequence of the rifampin resistance-determining regions of the rpoB genes from M. ulcerans and M. leprae. Only the nucleotides of wild-type (WT) or resistant (mutant R1, R2, and R3) M. ulcerans strains which differ from those in M. leprae are shown; identical nucleotides are denoted by periods. Note the amino acid changes Ser to Phe at position 416 and His to Tyr at position 420.](image)

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after the start of treatment, and only a slight persisting inflammation was observed in the tissues until the end of the experiment. This was probably a sequella of the extensive inflammatory lesions seen at the beginning of the infection.

It has been known since 1947 (27) that monotherapy with streptomycin and subsequently with other antituberculosis drugs results in the occurrence of drug-resistant mutants. Rifampin is no exception, and many studies have shown that resistant strains can be selected after tuberculous or leprosy is treated with rifampin alone (17).

After 3 months of experimental treatment of 30 infected mice with rifampin, followed by another 3 months of clinical observation, we isolated two strains (strains R1 and R2) for which the rifampin MIC was increased more than eightfold. The probability of a relapse by use of such a protocol was 6.7%. Among the 107 to 108 M. ulcerans bacilli inoculated, at least 1 bacillus was resistant to rifampin. This rate is close to those observed for M. tuberculosis and M. leprae. In early lesions in the edematous form or in small ulcer active lesions there can be 107 to 108 bacilli/g of tissue (K. Asiedu, B. Carbonnelle, J. Grosset, and M. H. Wansbrough-Jones, unpublished data). This indicates that such mutants can be found in these lesions and that the resistant bacilli could be selected by the use of rifampin alone.

The mechanism of action of rifampin was first studied in Escherichia coli (16) and was confirmed in other species. Rifampin targets the β subunit of DNA-dependent RNA polymerase, encoded by the rpoB gene (14), and resistance is due to missense mutations that occur in a highly conserved region of the gene. This phenomenon has been observed in M. tuberculosis, M. leprae, and other pathogenic mycobacteria (24). The comparative study of the rifampin resistance-determining region amplified from wild-type strain 897 and resistant strains R1 and R2 has shown that a point mutation is present in both R1 and R2. Serine 416 is thus replaced by phenylalanine, a voluminous and more hydrophobic amino acid. In strain R2, histidine is replaced by tyrosine. These changes have also been described in rifampin-resistant mutants of M. leprae and M. tuberculosis. Because of these substitutions the antibiotic can no longer bind to its target (9).

In M. tuberculosis, depending on the position of the mutation and the nature of the substituted amino acid, wide variations in the MIC are found (15). For strains R1 and R2, the MIC has increased to 8 μg/ml, which is a moderate level of resistance, whereas for strain R3, the MIC was 32 μg/ml, which corresponds to a high level of resistance. This modification did not affect the pathological effects of the resistant strains compared to that of the wild-type strain but led to clinical inefficacy, since 80% of the mice inoculated with strains R1 and R2 died, even with 90 days of treatment with rifampin. Modifications other than rifampin sensitivity were not observed in strains R1 and R2. Their physiological properties were the same as those of wild-type strain 897, and no other changes in their behaviors were observed, which is unlike the situation for certain resistant strains of E. coli (10).

In conclusion, the use of rifampin monotherapy of mice infected with M. ulcerans led to the isolation of resistant strains after 3 months of treatment. This result confirms the necessity of using a combination of different antibiotics for the treatment of Buruli ulcer to avoid the emergence of resistant strains in humans.

ACKNOWLEDGMENTS

We thank B. Denizot and V. Moreau-Lherbette for technical assistance. We are also grateful for a predoctoral fellowship from the Association Française Raoul Follereau (to L.M.). This work was supported by a grant from the Raoul Follereau Association.

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