***Mycobacterium leprae* isolates from different sources have identical sequences of the spacer region between the 16S and 23S ribosomal RNA genes**

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To test for genotypic variations between different isolates of *Mycobacterium leprae*, the causative agent of leprosy, the 282 bp spacer region between the 16S and 23S rRNA genes was amplified using PCR, and submitted to single-strand conformation polymorphism (SSCP) analysis. The procedure was optimized using four modified spacer fragments, containing mutations at one, three, four and six positions, respectively. Seventy-five *M. leprae* isolates from different sources, including isolates from leprosy patients, healthy individuals, armadillos and mouse footpads were identical in the SSCP analysis. DNA sequencing and restriction enzyme analysis performed on four and 40 samples, respectively, confirmed the results obtained with SSCP analysis.

Keywords: *Mycobacterium leprae*, 16S/23S rRNA spacer, single-strand conformation polymorphism

INTRODUCTION

Leprosy, caused by *Mycobacterium leprae*, is still a major health problem in many developing countries. Unfortunately it is very difficult to identify sources of infection because of the very long incubation time (Fine, 1982) and because *M. leprae* strains from different sources cannot be differentiated. Strain differentiation, as carried out for other mycobacteria, by phenotypic and chemotaxonomic features failed for *M. leprae* because of its inability to grow *in vitro*. Previous studies (Clark-Curtiss & Walsh, 1989; Williams *et al.*, 1990) used methods that require large amounts of purified bacteria, cultured through passage in the nine-banded armadillo (*Dasypus novemcinctus* Linn.). We chose a methodology which does not require large amounts of purified bacteria and is directly applicable to patient samples: *M. leprae* DNA was amplified using PCR and the resulting DNA fragments were analysed by single-strand conformation polymorphism (SSCP) analysis for possible differences (Orita *et al.*, 1989a). The 16S/23S ribosomal spacer region was chosen because it was found to be variable in other bacteria (Barry *et al.*, 1991; McLaughlin *et al.*, 1993; Matar *et al.*, 1993; Gürtler, 1993).

METHODS

***M. leprae* samples.** Seventy-five *M. leprae* samples were collected; 30 samples, directly taken as skin biopsy specimens (17) or as nasal swab specimens (13), came from multibacillary leprosy patients. Of these patients, two resided in Africa, 10 in Europe as immigrants from South-America and 18 in Asia. Twenty-three samples (nasal swab specimens) were collected from healthy individuals from Asia, who were found to carry *M. leprae* in their noses (Klatser *et al.*, 1993; de Wit *et al.*, 1993), but had not been diagnosed as leprosy patients. Fourteen isolates originated from armadillos: three of them were naturally infected, and 11 were experimentally infected with bacteria from multibacillary leprosy patients. Of these patients, seven were from Asia, two from Africa and two from North-America, and the bacteria had been grown for one, two or three passages in armadillos. Eight isolates originated from footpads of nude mice: one of which had been inoculated with *M. leprae* from a naturally infected Mangabey monkey from Africa and seven with bacteria from multibacillary patients from Africa. Of this latter group, six isolates had been cultured in armadillos and one in a nude mouse before inoculation into the mouse footpad.

Patients had been classified as multibacillary, when the bacterial index (BI) was > 0 , based on the enumeration of acid-fast bacteria of skin slit smears (Ridley, 1977).

Purified chromosomal *M. leprae* DNA was kindly supplied by Dr M. J. Colston as part of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. This DNA served as reference in all experiments.

Control samples. To confirm the specificity of the PCR, DNA

Abbreviation: SSCP, single-strand conformation polymorphism.

from human placenta, a non-infected armadillo, a mouse, *M. bovis* BCG, *M. intracellulare*, *M. fortuitum*, *M. kansasii*, *M. scrofulaceum*, *M. gordonae*, *M. vaccae* and from an armadillo-derived mycobacterium (ADM) were used. The origin of the mycobacterial strains was described by Hartskeerl *et al.* (1989).

Preparation of samples for PCR. Skin biopsy and nasal swab specimens were incubated for 18 h at 60 °C in 100 µl 10 mM Tris/HCl, pH 8.0, containing 1 mg proteinase K ml⁻¹ and 0.05 % (v/v) Tween 20. *M. leprae* bacteria were isolated from the liver (six), spleen (five) and leproma (three) from infected armadillos (Rodde *et al.*, 1992). Chromosomal DNA was extracted from the bacteria, as described previously (Hartskeerl *et al.*, 1989). Mouse footpads were incubated for 18 h at 37 °C in 500 µl 10 mM Tris/HCl, pH 8.0, containing 10 mg collagenase A ml⁻¹ (Boehringer Mannheim). The skeleton was removed from the resulting tissue suspension, which was then incubated for 18 h at 60 °C with proteinase K/Tween 20 at final concentrations of 1 mg ml⁻¹ and 0.05 % (v/v), respectively.

PCR. Amplification of the 16S/23S ribosomal spacer region was performed by two PCR runs using nested sets of primers, designed according to the sequence determined by Liesack *et al.* (1990a, b). The first set of primers was from a conserved region of the 16S rRNA gene (position 1512–1529) and from an *M. leprae*-specific region of the 23S rRNA gene (position 747–768). These primers generated a 1089 bp fragment, which was used as template for the second PCR, using primers from positions 1531–1548 of the 16S, and 1–18 of the 23S rRNA gene, respectively. The final product was a 318 bp fragment containing the complete 282 bp spacer region.

Both PCR runs were performed in reaction mixtures containing 10 mM Tris/HCl, pH 9.6, 7.5 mM MgSO₄, 50 mM NaCl, 0.01 % (w/v) gelatin, 1 mM each of the deoxynucleotides dATP, dCTP, dGTP and dTTP, 50 pmol of each primer, 10 % (v/v) DMSO and 2.5 U *Taq* DNA polymerase (Perkin Elmer). To avoid amplification of contaminating DNA samples, the PCR reaction mixtures were prepared in a DNA-free area separate from template preparation and amplification. Templates were added with positive displacement pipettes to further decrease the risk of carry-over contamination.

Because SSCP analysis requires DNA fragments with a high degree of purity, the PCR profile for both runs was performed according to the 'touchdown'-principle (Don *et al.*, 1991). The first 10 cycles were performed with an annealing temperature of 10 °C above the melting temperature (T_m) of the primers; the annealing temperature was then decreased by 1 °C every second cycle to the T_m of the primers, 66 °C for PCR-1 and 55 °C for PCR-2. Then 10 additional cycles were performed at this temperature, giving a total of 40 cycles.

Different amounts of template DNA were used for the first amplification and the products were separated by electrophoresis on a 2 % (w/v) agarose gel and visualized by ethidium bromide staining. Samples giving a distinct 1089 bp fragment similar in intensity to the amplification product obtained from 250 fg of the reference DNA were diluted 1:10 000 and used for the second PCR. The 318 bp fragments were isolated from 2 % agarose gels, using the GlassMax DNA Isolation System (Life Technologies) and dissolved in 50 µl distilled water. The purity of the 318 bp fragments was checked by electrophoresis of 10 µl of the GlassMax eluate on a 6 % (w/v) polyacrylamide gel in 90 mM Tris/acetate buffer, pH 8.0, containing 2 mM EDTA (TAE). DNA in the gels was visualized by a Silver Stain (Bio-Rad).

Construction of the modified 318 bp fragments. To determine the best conditions for the SSCP procedure, we constructed

four modified 318 bp fragments by PCR mutagenesis using four primers for each modification (Higuchi, 1990). The 1089 bp fragment amplified from *M. leprae* reference DNA, served as target for the mutagenesis. All modifications were chosen arbitrarily, both with respect to their position in the spacer region and to the number of nucleotides involved. All modifications introduced a restriction site into the 282 bp sequence of the spacer region (numbering as published by Liesack *et al.* (1991)): a 1 bp change at position 144 introduced a *Hae*III site (GGCT to GGCC); a change of 3 bp at position 220, 222 and 223 resulted in a *Pvu*I site (CGTTGT to CGATCG); 4 bp altered at position 158–161 resulted in another *Hae*III site (TTTG to GGCC) and finally a mutation of 6 bp at position 82–87 introduced an *Eco*RI site (TGCGCA to GAATTC).

The non-modified fragment and the four mutated fragments were cloned using the TA-vector pCRII and *Escherichia coli* InvαF' competent cells (Invitrogen). Digestion with the site-respective restriction enzymes confirmed the modifications of the cloned fragments.

SSCP. The conditions for SSCP were optimized using the 318 bp fragments amplified from the five recombinant clones. Five microlitres denaturing agent, 20 mM NaOH in dimethylformamide (DMF), was added to 5 µl of sample, followed by 10 min incubation at 100 °C and quenching on ice. Then 5 × sample loading buffer, either 5 × TAE, containing 40 % (w/v) sucrose, 0.25 % (w/v) bromophenol blue and 0.25 % (w/v) xylene cyanol, or the same solution with the addition of 50 % (v/v) glycerol was added. Samples were applied to either a 6 % (w/v) polyacrylamide or a Hydrolink-Mutation Detection Enhancement (MDE) gel (AT Biochem, Malvern, Pennsylvania, USA). Dimensions of both types of matrices were: 150 × 120 × 1 mm. Electrophoresis was performed in TAE, at a constant voltage of 200 V for 6 h at room temperature, when 6 % (w/v) polyacrylamide gels were used. MDE gels were run at a constant voltage of 200 V for 20 h at room temperature. No special measures were taken to achieve a uniform gel temperature. DNA on both types of gel was visualized by a Silver Stain (Bio-Rad).

The 318 bp fragments of the *M. leprae* isolates were denatured by the method described above. Sample loading buffer without glycerol was added and samples were subsequently applied onto an MDE gel. Electrophoresis and detection of DNA was performed as described above.

Restriction enzyme analysis and DNA sequencing. As additional tests to monitor for variations within the sequence of the spacer region, restriction enzyme analysis and DNA sequencing were performed on a number of 318 bp fragments. The restriction enzymes were selected on the basis of the known sequence of the spacer region (Liesack *et al.*, 1991). To monitor for mutations that resulted in disappearance of known restriction sites, we selected 11 enzymes having recognition sites in the sequence of the spacer region, i.e. *Ava*II, *Avi*II, *Dde*I, *Dra*II, *Fok*I, *Hba*I, *Hpa*II, *Mbo*II, *Nsp*II, *Sau*I and *Sau*96I. To monitor for mutations that resulted in the generation of new restriction sites, we selected 12 enzymes not having a recognition site in that sequence, i.e. *Ava*I, *Bmy*I, *Eco*RI, *Eco*RV, *Hae*II, *Hae*III, *Hin*FI, *Mbo*I, *Mva*I, *Sau*3AI, *Sma*I and *Xba*I. Using these two groups of enzymes, we tested 69 (24 %) nucleotides for possible mutation. A 10 µl sample of the GlassMax-purified 318 bp fragment was submitted to restriction enzyme analysis. Each digestion was performed under conditions as indicated by the manufacturer for the respective enzyme. Restriction patterns were analysed by electrophoresis on a 2 % (w/v) agarose gel, containing 50 µg ethidium bromide ml⁻¹. Forty *M. leprae*

samples were analysed: 20 from leprosy patients (10 skin biopsy, 10 nasal swab specimens), 12 from healthy individuals, four from armadillos and four from mouse foot pads. The samples were chosen arbitrarily from each source.

For DNA sequencing, the 318 bp fragments were cloned into the TA-vector pCRII (Invitrogen). Recombinant plasmids were isolated using Qiagen Tip 100 (Diagen). DNA sequencing was performed with an upstream and a downstream primer based on the sequence of the multiple cloning site of the vector, using the T7 sequencing kit (Pharmacia). Four *M. leprae* samples were analysed: one from a leprosy patient (nasal swab specimen), one from a healthy individual, one from an armadillo and one from a mouse footpad. From each source the sample was chosen arbitrarily.

RESULTS

Amplification by PCR

The specificity of the chosen oligonucleotide primers for amplification of the 282 bp *M. leprae* 16S/23S spacer region was confirmed using template DNA from several mycobacteria, mouse, armadillo and human placenta. No amplification product was observed with 250 fg or 1 ng template DNA for the first PCR. The only exception was *M. kansasii*, which gave a 318 bp amplification product with 1 ng, but not with 250 fg template DNA. All 75 *M. leprae* isolates gave a single 318 bp fragment.

SSCP analysis

The 318 bp fragments amplified from the five *in vitro*-generated recombinant clones were used to optimize the SSCP procedure. With polyacrylamide gels, 4- and 6-base changes were detectable as band shifts; addition of glycerol to the sample loading buffer also produced a band shift for the fragment with three mutations. The single mutation also became apparent as a very clear shift in

electrophoretic mobility when polyacrylamide gels were substituted by MDE gels (Fig. 1, lane 7). Glycerol did not influence the results obtained with this type of gel (results not shown). The 318 bp fragments from all 75 *M. leprae* isolates were analysed on MDE gels. None of them showed a shift in the electrophoretic mobility of the two single-stranded fragments. This is illustrated for five isolates in Fig. 1 (lanes 1–5).

Restriction enzyme analysis and DNA sequencing

The occurrence of variations within the sequence of the spacer region was also investigated by restriction enzyme analysis and DNA sequencing on a number of 318 bp fragments.

When incubated with the 11 enzymes having a recognition site in the sequence of the spacer region, the 40 investigated *M. leprae* samples revealed the same restriction patterns. The sizes of the fragments in the restriction patterns were as calculated from the positions of the recognition sites in the sequence of the spacer region as published previously (Liesack *et al.*, 1991) (results not shown). No digestion of the 318 bp fragments was observed when incubated with any of the 12 enzymes that did not have a recognition site in the sequence of the spacer region (results not shown).

DNA sequencing revealed that the nucleotide sequence of the 318 bp fragments from the four investigated *M. leprae* isolates was identical to the sequence of both the reference fragment and the spacer region as published previously (Liesack *et al.*, 1991) (results not shown).

DISCUSSION

In this paper we report the results of SSCP analysis performed on the 282 bp 16S/23S ribosomal spacer region of 75 *M. leprae* isolates. The specificity for *M. leprae* of one of the oligonucleotides from within the 23S rRNA gene (positions 747–768), was determined by Liesack *et al.* (1991). The specificity of the PCR amplification for *M. leprae* was confirmed using various controls, including DNA from several mycobacterial species. The use of these PCR amplified fragments made it possible to use samples directly from patients and from healthy *M. leprae*-infected individuals (Klatser *et al.*, 1993; de Wit *et al.*, 1993). Very pure samples, essential for SSCP analysis, were obtained using high temperatures for the initial annealing steps and a two-step amplification protocol using nested primers. The results of SSCP analysis depend very much on the choice of the optimal gel running conditions for a particular DNA fragment (Orita *et al.*, 1989a, b; Dockhorn-Dworniczak *et al.*, 1991; Hayashi & Yandell, 1993). Therefore, our conditions were optimized specifically for the amplified *M. leprae* spacer fragment using *in vitro*-constructed mutant derivatives of this fragment. Substituting Hydrolink MDE gels for polyacrylamide allowed reproducible distinction of all mutant fragments from each other and from the original sequence. These conditions would probably detect most, but not neces-

Fig. 1. SSCP analysis of 318 bp amplification products from various *M. leprae* samples applied on a MDE gel. Lanes: 1, isolate from an armadillo, experimentally infected with bacteria from a patient with multibacillary leprosy (MB), Asia; 2, nasal swab from a MB patient, Asia; 3, nasal swab from a healthy person, Asia; 4, skin biopsy specimen from a MB patient, Asia; 5, isolate from a mouse footpad inoculated with bacteria from a MB patient, Africa; 6, 318 bp from the cloned reference fragment; 7, 318 bp fragment containing 1 mutation; 8, 318 bp fragment containing 3 mutations; 9, 318 bp fragment containing 4 mutations; 10, 318 bp fragment containing 6 mutations; 11, WHO reference, genomic DNA.

arily all the possible base changes in the amplified fragment (Orita *et al.*, 1989a). The effectiveness of SSCP analysis for the high G+C *Mycobacterium* DNA has been demonstrated by Telenti *et al.* (1993), who detected all 61 single nucleotide mutations in a set of 66 rifampicin-resistant *Mycobacterium tuberculosis* strains.

No polymorphism could be detected among the 75 *M. leprae* samples which were probably representative for all the *M. leprae* strains present in the world. They were from four continents, sick and healthy infected subjects, armadillos and a monkey. Some of the samples were taken directly from the patients, others were first subcultured in armadillos and mice.

Further evidence for the lack of sequence polymorphism in the 282 bp spacer region was obtained from the digestion of fragments with several restriction endonucleases which would have detected mutations in 24% of the nucleotides, and from the sequencing of four fragments chosen from different sources.

The apparent 100% sequence conservation of the *M. leprae* 16S/23S ribosomal spacer region seems remarkable because in other organisms this region was useful for strain differentiation (Barry *et al.*, 1991; McLaughlin *et al.*, 1993; Matar *et al.*, 1993; Gürtler, 1993). It is also variable between different strains of mycobacteria and has been used for the establishment of phylogenetic relationships (Ji *et al.*, 1994). One possible explanation for sequence conservation may be that *M. leprae*, like other slow growing mycobacteria, contains only one *rrn* operon (Sela *et al.*, 1989), while most other bacteria contain several, maybe allowing mutations to persist till compensating mutations neutralize deleterious effects. Other possible reasons for this conservation were put forward by Clark-Curtiss & Walsh (1989).

The results from our study confirm the general view that all *M. leprae* isolates are very similar (Clark-Curtiss & Walsh, 1989; Honore & Cole, 1993; Williams *et al.*, 1990). Some differences between *M. leprae* isolates have, however been discovered in the growth rate (Shepard & McRae, 1971) and in the sequence of the EF-Tu gene (Silbaq & Bercovier, 1993).

There is still a need for a method for *M. leprae* strain differentiation that would greatly help epidemiological studies, including the question of whether the different clinical manifestations of leprosy are a consequence of differences in the bacteria or the host. To determine whether genotypic variation within the species *M. leprae* does exist in other, so far undetermined parts of the genome, we will conduct further studies in which we will employ methods that give reproducible fingerprints of larger parts of the genome (Haas *et al.*, 1993). Until results from such tests are available, we remain uncertain about the existence of different strains of *M. leprae*.

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