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Emergence of Group A Streptococcus Strains with Different Mechanisms of Macrolide Resistance

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The mechanisms of resistance to macrolides in seven group A streptococcal (*Streptococcus pyogenes*) isolates that were the cause of pharyngitis in children who were unsuccessfully treated with azithromycin (10 mg/kg of body weight/day for 3 days) were evaluated. All posttreatment strains were found to be genetically related to the pretreatment isolates by random amplified polymorphism DNA analysis and pulsed-field gel electrophoresis. Two isolates had acquired either a *mef*(A) or an *erm*(B) gene, responsible for macrolide efflux and ribosomal modification, respectively. Three isolates displayed mutations in the gene encoding the L4 ribosomal protein that is part of the exit tunnel within the 50S subunit of the bacterial ribosome. In the two remaining posttreatment strains, the mechanisms of macrolide resistance could not be elucidated.

The recommended treatment for group A *Streptococcus* (GAS) pharyngitis, a 10-day course of penicillin, was established 50 years ago and remains the "gold standard."

However, for patients hypersensitive to β-lactam antibiotics and in whom therapy with these drugs fails, macrolides are often the recommended substitute. Azithromycin is an azalide antibiotic chemically related to erythromycin and has a long half-life and excellent tissue penetration. Short-course azithromycin therapy has been reported to be effective for the eradication of oropharyngeal GAS (14).

In a prospective, comparative, randomized, multicenter trial that was conducted between November 1997 and July 1998 and that involved 350 children with GAS pharyngitis, we observed better eradication rates on days 14 and 30 after a 3-day course of therapy with azithromycin at 20 mg/kg of body weight/day than after a 3-day course of therapy with azithromycin at 10 mg/kg/day. In the per protocol analysis, the failure rates on day 14 were 57 of 135 (42.2%) in the 10-mg/kg treatment arm and 8 of 139 (5.8%) in the 20-mg/kg treatment arm (8). Furthermore, analysis of bacterial isolates by random amplified polymorphic DNA (RAPD) analysis revealed seven cases of bacteriological treatment failure: azithromycin MICs for genetically related pre- and posttreatment GAS strains increased after treatment with this antimicrobial at 10 mg/kg/day for 3 days. In contrast, azithromycin MICs were not increased for any of the strains from any of the patients with treatment failure in the group treated with azithromycin at 20 mg/kg/day (8). The known mechanisms of macrolide resistance in streptococci are modification of the ribosomal target by a methylase encoded by *erm* genes (13, 20) and a macrolide-specific efflux mechanism encoded by the *mef*(A) gene (7). Mutation of the ribosomal target of macrolides is a rare resistance mechanism in streptococci and has been reported in only a few clinical pneumococcal isolates (21).

In the study described here we investigated the mechanisms of macrolide resistance among the GAS strains that were the cause of bacteriological treatment failures in the group treated with azithromycin at 10 mg/kg/day.

MATERIALS AND METHODS

Bacterial isolates. GAS isolates were obtained by swabbing of the throat on day 0 (pretreatment isolate [V1]) and day 14 or day 30 (posttreatment isolates [V2]) after the onset of low-dose azithromycin treatment in children. All GAS isolates were associated with bacteriological treatment failure (persistence or relapse) (6, 10), and the azithromycin MICs for the posttreatment isolates had increased. The isolates were identified as GAS by colony morphology, beta-hemolysis on blood agar, and a commercial agglutination technique (Murex Diagnostics, Dartford, United Kingdom).

MIC determination. The MICs of erythromycin, azithromycin, josamycin, and clindamycin were determined by the agar dilution method with Mueller-Hinton medium supplemented with 5% defibrinated sheep blood (15). The plates were incubated overnight at 35°C in ambient air.

Molecular analysis. RAPD analysis and pulsed-field gel electrophoresis (PFGE) were used to compare pre- and posttreatment GAS isolates in order to distinguish between persistence-relapse and the acquisition of new strains (6, 10). RAPD analysis and PFGE were based on previously described methods (2, 10). For PFGE, *Sma*I and *Sfi*I chromosomal digests were separated by using a CHEF-MAPPER apparatus (Bio-Rad, Marnes la Coquette, France) with a switch time of 0.85 to 35.38 s for 22 h and 35 min at a 120° angle with a voltage gradient of 6 V/cm at 14°C. The DNA size standard was a bacteriophage lambda DNA ladder (Bio-Rad). The PFGE banding patterns were compared visually. Strains were considered genetically distinguishable if their restriction patterns differed by three or more bands (23).

Detection of erythromycin resistance genes. All erythromycin-resistant isolates were screened for erythromycin resistance genes. The *erm*(B), *erm*(A) subclass *erm*(TR), and *mef*(A) genes were detected by PCR amplification as described previously (3). *Streptococcus pyogenes* 02C1061, *S. pyogenes* 02C1110, and *S. pyogenes* 02C1064 were used as positive PCR controls for the *erm*(B), *erm*(A) subclass *erm*(TR), and *mef*(A) genes, respectively (3).

DNA from PFGE was transferred to nitrocellulose membranes (Hybond-N^+; Amersham, Little Chalfont, England) by vacuum blotting. DNA was hybridized with a digoxigenin-labeled probe of 616 bp obtained by PCR amplification of the *erm*(B) gene from reference strain *S. pyogenes* 02C1061 containing the *erm*(B) gene (3). Hybridization and colorimetric detection were performed as recommended by the manufacturers (Roche Molecular Biochemicals, Meylan, France).

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TABLE 1. Oligonucleotides used for amplification of *rrl* gene (23S rRNA)

Primer designation	Primer sequence (5′ to 3′)[a]	Position[b]	Product size (bp)
23SCPU1	+ GTTAATAAGGGCGCACGG	7–24	247
23SCPL1	− GCTCGCCGCTACTAAGG	237–253	
23SCPU2	+ TTAGTAGCCGCAGGAAGAG	192–210	336
23SCPL2	− GTAGGCACACGGTTTCAGG	509–527	
23SCPU3	+ GAACCAGTACCGTGAGGG	452–469	384
23SCPL3	− TAGCCCTAAAGCTATTTCGG	816–835	
23SCPU4	+ GATGACTTGTGGGTAGCGG	760–778	381
23SCPL4	− GCCCCGGTACATTTTCGG	1123–1140	
23SCPU5	+ GGTTGCCCAGACAACTAGG	1038–1056	367
23SCPL5	− GTACAGGAATATCAACCTG	1386–1404	
23SCPU6	+ GCTCGTCCGCCCTGGG	1324–1339	377
23SCPL6	− TCTCCCGAAGTTACGGGG	1683–1700	
23SCPU7	+ GTACCGCAAACCGACACAG	1601–1619	406
23SCPL7	− GTCTCTCGTTGAGACAGTG	1988–2006	
23SCPU8	+ GACCCGCACGAAAGGCG	1959–1975	401
23SCPL8B	− GTAGCTCTCGCAGTCAAG	2342–2359	
23SCPU9	+ GTTCCCTCAGATTGGTTGG	2290–2308	386
23SCPL9	− GCGTGCCGCTTTAATGGG	2558–2575	
23SCPU10	+ GTAGTCGGTCCCAAGGG	2529–2545	372
23SCPL10	− ATAAGTCCTCGAGCGATTAG	2880–2900	

[a] +, sense primer; −, antisense primer.
[b] *E. coli* numbering.

Detection of mutations in the ribosomal target of macrolides. The nucleotide sequences of the 23S rRNA and L4 and L22 ribosomal proteins in *Escherichia coli* were obtained from The Institute for Genomic Research website (http://www.tigr.org.), and homologs in GAS were detected by using BLAST software (9). Specific oligonucleotide primers were then designed. We amplified a portion of the *rrl* gene for domain II from nucleotides (nt) 580 to 852 (*E. coli* numbering) with primers 5′-CGGCGATTACGATATGATGC-3′ and 5′-CTCTAATGTCGACGCTAGCC-3′ and two fragments of domain V of 23S rRNA (nt 1990 to 2134 and nt 2331 to 2769) with two pairs of primers (primers 5′-CTGTCTCAACGAGAGACTC-3′ and 5′-CTTAGACTCCTACCTATCC-3′ and primers 5′-GTATAAGGGAGCTTGACTG-3′ and 5′-GGGTTTCACACTTAGATG-3′). The entire L22 (*rplV*) and L4 (*rplD*) genes were amplified with pairs of primers (primers 5′-GCTGACGACAAGAAAACACG-3′ and 5′-GCCGACACGCATACCAATTG-3′ and primers 5′-CAAGTCAGGAGTTAAAGCTGC-3′ and 5′-CAACTTCGAAAGTGTATTTGCC-3′, respectively). The three amplified *rrl* fragments (two for domain V and one for domain II) included bases critical for erythromycin resistance (G2057, A2058, A2059, A2062, G2505, C2611, A754, and A752). PCR products were sequenced by the rhodamine dye terminator method with an ABI Prism 377 sequencer (Perkin-Elmer Corp., Norwalk, Conn.). For the two pairs of pre- and posttreatment isolates of strains 133 and 233, the sequences of the *rrl* genes were obtained as follows. Ten overlapping fragments were amplified with the primers shown in Table 1 and sequenced. Amplicons were also analyzed by single-strand conformation polymorphism analysis as described previously (5). Briefly, aliquots of amplified fragments were heat denaturated, and the single-strand PCR products were then separated by nondenaturant polyacrylamide gel electrophoresis. Fragments bearing mutations could be distinguished from wild-type fragments on the basis of different electrophoretic mobilities.

RESULTS

Using RAPD analysis and PFGE, we found that the seven paired pre- and posttreatment GAS isolates associated with persistence or relapse were genetically related (Fig. 1 and 2). Two of the seven pairs of strains were of the same PFGE type, although the patients were epidemiologically unrelated. For patient 508, the DNA of the posttreatment strain could not be analyzed by PFGE with *Sma*I, despite repeated attempts. Macrolide MICs for the paired GAS strains are shown in Table 2. All pretreatment isolates were sensitive to 14-, 15-, and 16-membered-ring macrolides and clindamycin, while the posttreatment isolates showed different levels of resistance accord-

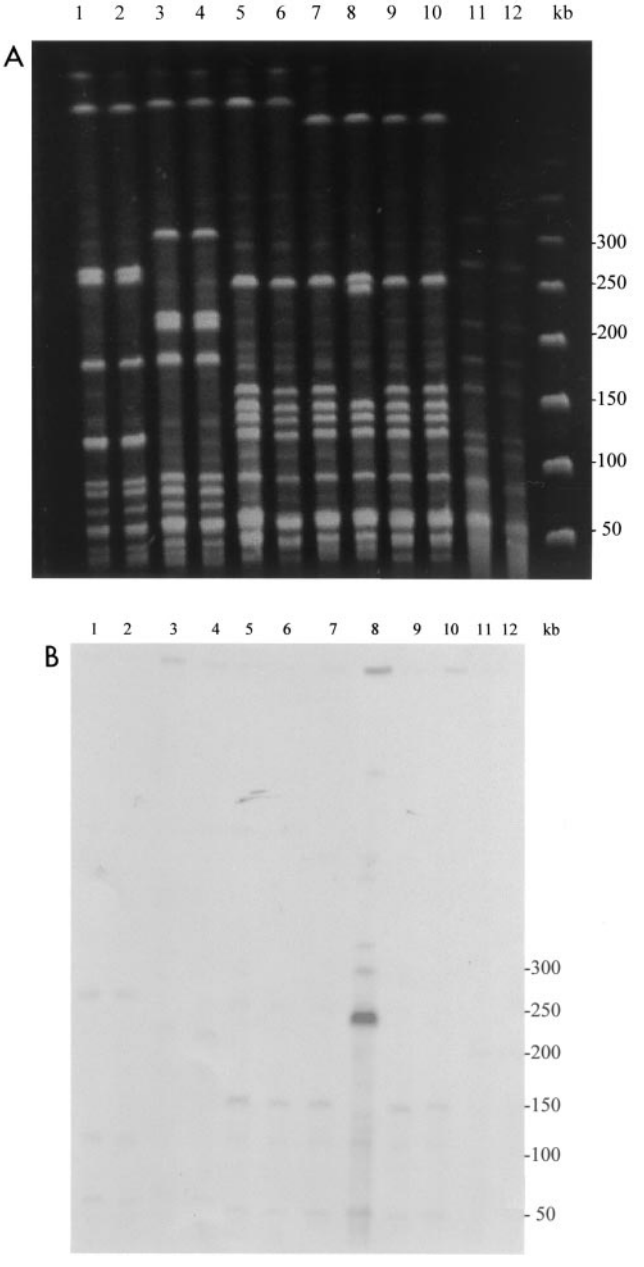

FIG. 1. (A) PFGE patterns of the pre- and posttreatment GAS isolates digested with *Sma*I. Lane 1, isolate 11 V1; lane 2, isolate 11 V2; lane 3, isolate 124 V1; lane 4, isolate 124 V2; lane 5, isolate 390 V1; lane 6, isolate 390 V2; lane 7, isolate 286 V1; lane 8, isolate 286 V2; lane 9, isolate 133 V1; lane 10, isolate 133 V2; lane 11, isolate 323 V1; lane 12, isolate 323 V2. (B) Southern blot of the gel with an *erm*(B) probe. Lane 8, isolate 286 V2 shows a signal with the probe. The molecular sizes of the standards (in kilobases) are shown to the right of the gels.

TABLE 2. MICs of macrolides and clindamycin for the seven pre- and posttreatment GAS isolates by mechanism of resistance

Patient no.	GAS isolate[a]	Isolation date (mo/day/yr)	MIC (μg/ml)				Mechanism of resistance
			Erythromycin	Azithromycin	Josamycin	Clindamycin	
11	11 V1	11/19/97	0.064	0.125	0.5	0.125	
11	11 V2	12/03/97	0.5	2	1	0.125	L4 mutation
124	124 V1	01/12/98	0.064	0.25	0.5	0.064	
124	124 V2	01/23/98	0.5	1	1	0.064	L4 mutation
390	390 V1	05/05/98	0.064	0.25	0.5	0.125	
390	390V2	05/23/98	1	2	2	0.125	L4 mutation
286	286 V1	03/23/98	0.064	0.125	0.5	0.125	
286	286 V2	04/06/98	>128	>128	>128	>128	*erm*(B)
133	133 V1	01/15/98	0.064	0.125	0.5	0.125	
133	133 V2	01/27/98	8	8	2	0.125	Unknown
323	323 V1	04/08/98	0.064	0.125	0.5	0.125	
323	323 V2	05/11/98	32	>128	128	1	Unknown
508	508 V1	06/05/98	0.064	0.25	0.5	0.125	
508	508 V2	06/16/98	8	8	0.5	0.125	*mef*(A)

[a] V1, isolate obtained before treatment; V2, isolate obtained after treatment.

ing to the antibiotic tested. The azithromycin MIC for the posttreatment isolate of strain 286 was >128 μg/ml, whereas the azithromycin MIC for the isolate was 0.12 μg/ml before treatment. PCR and Southern hybridization showed that the azithromycin resistance in this strain was due to acquisition of an *erm*(B) gene. The *erm*(B) gene was located on a chromosomal fragment of 250 kb which resulted from the insertion of a DNA sequence of ~90 kb in a ~160-kb *Sma*I fragment of the pretreatment isolate's DNA (Fig. 1B). For the posttreatment isolate of the pair of isolates from patient 508, acquisition of a *mef*(A) gene, as identified by PCR, resulted in resistance to azithromycin (MIC, 8 μg/ml). For pairs of isolates of three strains (strains 11, 124, and 390), azithromycin MICs increased from 0.125 or 0.25 μg/ml to 1 or 2 μg/ml. No *erm* or *mef* genes were detected in the three posttreatment isolates of these strains. However, sequence analysis of structures belonging to the macrolide binding site revealed mutations in a highly conserved region of ribosomal protein L4 ($_{61}$KPWRQKGTGRA R_{72}). The mutations consisted of a WR deletion at positions 64 and 65, an RA insertion after position 72, and a TG deletion at positions 69 to 70 (*S. pyogenes* numbering) in posttreatment isolates of strains 11, 124, and 390, respectively. Since no mutation was found in posttreatment isolates of strains 133 and 323, the entire *rrl* genes from these strains and their parents were sequenced. The DNA sequences were identical to those of the respective parent strains. Since *S. pyogenes* harbors six copies of genes for rRNAs, we could not exclude the possibility that only a minority of copies were mutated and therefore not detected by sequencing of genomic DNA as a bulk. To explore this possibility, we used PCR–single-strand conformation polymorphism analysis. In *Streptococcus pneumoniae*, which contains four copies of the *rrl* genes, this technique allowed investigators to distinguish a single wild-type copy from three mutated copies on the basis of heterogeneous electrophoretic profiles (5). The migration profiles of the PCR products were homogeneous and identical for the parent and the resistant mutated strains. Therefore, no explanation for the azithromycin resistance of the posttreatment isolates of strains 133 and 323 was found.

DISCUSSION

Resistance to macrolides has been widely reported in GAS, although its incidence varies considerably among countries. A

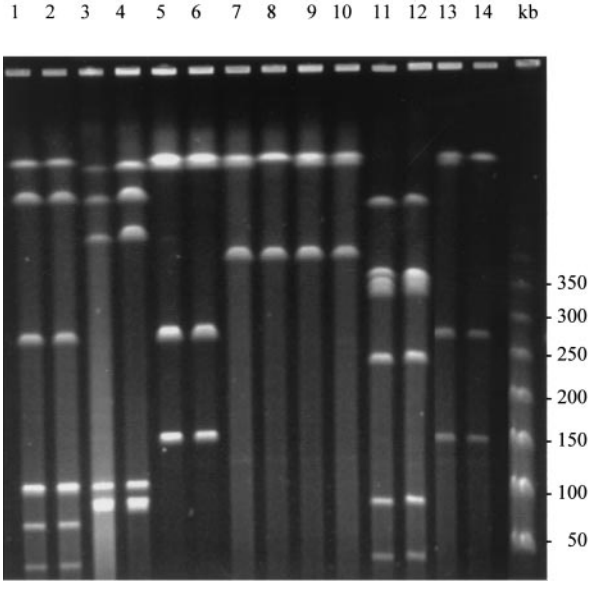

FIG. 2. PFGE patterns of the pre- and posttreatment GAS isolates digested with *Sfi*I. Lane 1, isolate 11 V1; lane 2, isolate 11 V2; lane 3, isolate 124 V1; lane 4, isolate 124 V2; lane 5, isolate 390 V1; lane 6, isolate 390 V2; lane 7, isolate 286 V1; lane 8, isolate 286 V2; lane 9, isolate 133 V1; lane 10, isolate 133 V2; lane 11, isolate 323 V1; lane 12, isolate 323 V2; lane 13, isolate 508 V1; lane 14, isolate 508 V2.

previous study from Finland demonstrated that a decrease in the incidence of macrolide resistance in *S. pyogenes* could be managed by limiting erythromycin use through a national program (19). However, the rate of use of newer macrolides with fewer daily dosages increased during the course of the study, which suggests that the overall numbers of patients treated with macrolides did not decrease (19).

Resistance to macrolides was reported to be associated with dissemination of clonal strains (24) but rarely with the in vivo acquisition of resistance during therapy. In our work, the number of GAS isolates (7 of 135) that developed resistance to macrolides after low-dose azithromycin treatment was strikingly high (8). This phenomenon was not observed in three recent clinical trials of azythromycin treatment in patients with tonsillopharyngitis (4, 16, 18). In two studies (4, 18) the susceptibilities of the posttreatment isolates were not reported. In the last study (16), no acquisition of resistance was observed. However, the large number of patients included in our study may account for the difference with the latter study.

The efficacy of treatment against streptococcal pharyngitis should be evaluated both clinically and bacteriologically (6). If microbiological treatment failure occurs, persistence or relapse due to the original GAS strain should be distinguished from recurrence due to acquisition of a new strain (6, 10). Genomic typing methods such as RAPD analysis and PFGE have been used to discriminate among *S. pyogenes* isolates (2, 10). We applied these techniques to the analysis of GAS strains isolated during a clinical trial of azithromycin therapy (10 or 20 mg/kg/day) for GAS pharyngitis in children. RAPD analysis and PFGE suggested that seven cases of microbiological treatment failure were due to GAS persistence or relapse, with acquisition of macrolide resistance after treatment with azithromycin at 10 mg/kg/day (8). However, we cannot exclude the possibility of the presence at the onset of the study of a double population of organisms, some of which had already acquired the gene for resistance, leading to the selection of the resistant organism by therapy.

In our study, among the seven cases of emergent azithromycin resistance, one was due to acquisition of a *mef* gene and another was due to the acquisition of an *erm* gene. The acquisition of a DNA fragment of about 90 kb bearing an *erm* gene by posttreatment strain 286 could correspond to acquisition of a mobile element or, rather, to the integration of a plasmid in the chromosome. Previous studies have shown that the commensal flora serves as a potential source of macrolide resistance determinants (1, 17). The location of *erm* on conjugative transposons and of *mef* on a transposon (12) might facilitate the circulation of these genes among the oropharyngeal flora under selective antibiotic pressure. Interestingly, we observed three cases of microbiological persistence or relapse with acquisition of L4 ribosomal mutations. So far, the only streptococcal species reported to have acquired resistance to macrolides by a mutation in 23S rRNA or ribosomal proteins is *S. pneumoniae*, both among clinical isolates (21) and among strains selected by multiple passages in the presence of azithromycin in vitro (22). Mutations in the gene encoding the L4 proteins of GAS strains and *S. pneumoniae* were clustered in an identical conserved region of the protein. In *E. coli*, L4 mutations perturb the three-dimensional structure of 23S rRNA at multiple sites and could therefore hypothetically prevent macrolide binding (11).

In the case of pneumococci and macrolides, the mutations detected in laboratory-derived mutants obtained with subinhibitory concentrations of azithromycin could be used to predict the mutations observed in clinical strains (22). The observation that repeated exposure to low concentrations of azithromycin selects resistant pneumococcal strains in vitro might help provide an understanding of the selection of GAS mutants in vivo. In this analysis, we found that three of seven clinical strains contained mutations in the gene encoding ribosomal protein L4, with modest increases in the MICs for strains detected. Similar mutations were not found among strains from patients in the 20-mg/kg azithromycin treatment arm with bacteriological treatment failures; either the L4 mutation and the resulting low MICs do not occur for a total dose of 60 mg, or the sample size for either treatment arm does not permit one to draw definitive conclusions. However, the higher dose of azithromycin gave significantly better bacterial eradication rates, thereby minimizing the chance for the emergence of resistance. Finally, no L22, L4, or 23S rRNA mutations were detected in two posttreatment strains; their mechanisms of resistance are still under investigation. Characterization of the mechanisms of resistance to macrolides, lincosamides, and streptogramin B antibiotics are important for the clinical management of antibiotic therapy and can provide clinicians with alternative treatment choices.

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