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Emergence during Unsuccessful Chemotherapy of Multiple Drug Resistance in a Strain of *Mycobacterium tuberculosis*

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Serial isolates of *Mycobacterium tuberculosis* were cultured from a patient who failed to respond to standard antituberculous chemotherapy. Isolates were cultured in March 1989, July 1989, December 1989 and May 1990. Each successive isolate was found to be resistant to a wider range of antituberculous drugs than its predecessors. The initial isolate was resistant to isoniazid and rifampin, the second isolate was also resistant to ethambutol, the third was also resistant to pyrazinamide, ansamycin (= rifabutin) and ofloxacin and the last isolate was also resistant to ciprofloxacin and sparfloxacin. All four isolates' bacteriophage typing profiles and DNA restriction fragment patterns determined by Southern blot hybridization using the IS*6110* /IS*986* probes and the new probe pTBN12 were concordant. It was concluded that this patient was persistently infected with a single strain of *Mycobacterium tuberculosis* which developed resistance to a number of families of drugs but did not show any significant change in typing patterns. The problem of acquired multiple drug resistance, particularly to fluoroquinolones and rifamycins, represents a new challenge in tuberculosis therapy.

Tuberculosis remains a major world health problem, with about one-third of humanity infected by *Mycobacterium tuberculosis*. Each year, *Mycobacterium tuberculosis* infections cause around 3 million deaths representing one-quarter of all preventable deaths (1). The US Public Health Service has set a goal for the elimination of tuberculosis from the USA by the year 2010 (2). Unfortunately, the AIDS pandemic is likely to interfere with this objective because of a resurgence of tuberculosis linked to AIDS/HIV infection (3), the emergence of multiple drug resistant *Mycobacterium tuberculosis* (4) and the increased treatment failure rates and associated mortality in HIV-infected patients (5). These reports underline the need for new initiatives in chemotherapy, epidemiology and infection control if the goal of eradication is to be achieved (6).

An important aspect of disease control is the ability to examine transmission patterns. Until now simple, highly discriminative typing systems have been unavailable for *Mycobacterium tuberculosis*. The development of bacteriophage typing in the 1970s, and more recently the development of chromosomal restriction fragment length polymorphism (RFLP) analysis by Southern hybridization, have provided the possibility of determining the identity and relatedness of *Mycobacterium tuberculosis* strains (7, 8). The emergence of drug resistance in a patient receiving chemotherapy provided an opportunity to study four successive isolates with these methods.

Materials and Methods

Case Report. A 32-year-old male from the Indian subcontinent was diagnosed with tuberculosis in 1983 and responded to a standard chemotherapy regimen of ethambutol, rifampin and isoniazid. Six years later, in March 1989, a year after a course of corticosteroids, he developed weight loss and fever. He did not have antibodies to HIV. His sputum had numerous acid-fast bacilli on microscopy and *Mycobacterium tuberculosis* was recovered on culture. He was given isoniazid, rifampin, ethambutol and pyrazinamide for two months and improved clinically, although his cultures remained positive. Because the March 1989 isolate (A) was resistant to isoniazid and rifampin, these two drugs were discontinued and replaced by ofloxacin (600 mg/day). Within a month the patient improved, his weight rose and his cough ceased. He was then discharged on treatment to a nursing home. In July 1989, in the absence of clinical

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Table 1: Drug susceptibility profile of four clinical isolates of *Mycobacterium tuberculosis* (A-D) obtained from the same patient in March 1989, July 1989, December 1989 and May 1990, respectively.

Drug	Breakpoint (µg/ml)	Susceptibility profile of isolate			
		A	B	C	D
Isoniazid	0.1	R	R	R	R
Isoniazid	0.5	R	R	R	R
Rifampin	1.0	R	R	R	R
Ethambutol	7.5	S	R	R	R
Ethionamide	10.0	S	S	S	S
Pyrazinamide	50.0	S	S	R	R
Ansamycin/Rifabutin	1.0	S	I	R	R
D-Cycloserine	30.0	S	S	S	S
Streptomycin	2.0	ND	S	S	S
Clofazimine	1.0	S	S	S	S
Ofloxacin	1.0	S	S	R	R
Ciprofloxacin	1.0	S	S	S	R
Sparfloxacin	0.25	ND	S	S	R

symptoms, *Mycobacterium tuberculosis* was cultured from a specimen of gastric aspirate (isolate B). This isolate was now resistant to ethambutol, and showed intermediate susceptibility to ansamycin. Following a clinical relapse with smear-positive sputum in December 1989 (isolate C), the patient was treated with isoniazid, ofloxacin and streptomycin (1 g/day for 45 days). However, as the isolate C was later found to be resistant to pyrazinamide, ansamycin and ofloxacin, the drug therapy was changed to isoniazid, clofazimine and ethionamide (pyrazinamide was not given after December 1989). There was transient improvement followed by relapse in May 1990 (isolate D). This last strain was resistant to both ciprofloxacin and sparfloxacin, although neither had been used. The patient showed progressive deterioration despite pneumonectomy and died in 1991.

Bacterial Isolation and Susceptibility Tests. The organisms were recovered on Löwenstein-Jensen medium at 37 °C and identified using standard methods (9). Drug susceptibility for 13 antibiotics was determined on 7H11 agar using the 1 % proportion method. The drugs and the concentrations used are listed in Table 1. In brief, *Mycobacterium tuberculosis* suspensions were made from Löwenstein-Jensen slopes by homogenizing bacteria in sterile distilled water using 2 mm glass beads. The optical density of the suspensions was adjusted to 0.15 at 650 nm, corresponding to approximately 10^8 viable organisms per ml as determined by plate counts. Appropriate dilutions were plated onto 7H11 agar containing fixed (breakpoint) concentrations of antibiotics and onto drug-free 7H11 agar. Colony counts were determined after 21 days of incubation at 37 °C. Organisms were regarded as susceptible if there were fewer than 1 % of survivors compared to the control plates. Precise MICs were determined by plating onto 7H11 agar containing serial dilutions of antibiotics. The MIC was defined as the lowest concentration of drug able to cause a 99 % reduction in the viable counts compared to the drug free controls (10, 11).

Increase in the resistance to fluoroquinolones was assessed both by determining MICs radiometrically as well as by comparing the bactericidal activity against successive *Mycobacterium tuberculosis* isolates using the Bactec 460-TB (12, Becton Dickinson, USA). The method used was the same as that described recently for screening activity of fluoroquinolones against *Mycobacterium tuberculosis* (10). Briefly, bacterial growth in a confined atmosphere was measured as a function of the ability of bacteria to catabolize ^{14}C-labelled palmitic acid in 7H12 broth, which resulted in the liberation of $^{14}CO_2$ expressed as a numerical value referred to as the growth index (GI), which ranged from 1 to 999. For radiometric determination of MICs, 0.1 ml of the suspension from a fresh primary culture of bacteria in 7H12 broth grown to a GI of 500 was injected into control and drug-containing vials. Daily GI values were recorded and compared with the GI of a 1:100-fold diluted control and results were interpreted when the GI of the 1:100 control reached a value of 30 or more. The MIC was defined as the minimal drug concentration resulting in a smaller change in the GI in the drug-containing sample compared with the 1:100 control.

For viable count determinations, 0.1 ml culture from Bactec vials was serially diluted to give 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions, and 0.1 ml each of these dilutions was plated onto 7H11 agar medium. The resulting colony forming units (cfu)/ml were enumerated after 21 days of incubation at 37 °C, and results were expressed as mean with standard error.

Bacteriophage Typing. The lytic profiles of the isolates A, B, C and D were compared using the bacteriophages and methods recommended by the World Health Organization (13). The bacteriophages, their host strains and the spot test dilutions are listed in Table 4. We have recently used this method to define five distinct lysotypes of *Mycobacterium tuberculosis,* namely A, AX, I, B and C, and have shown that lysotypes correlate with the geographic origin of patients (14, 15).

Labelling of Nucleic Acid Probes. Both RNA and DNA probes were labelled using a non-radioactive system (Boehringer Mannheim, Germany). A labelled RNA

probe of plasmid pTBN12 (8) was prepared from *Eco*RI digested plasmid using T3 RNA polymerase and digoxigenin-UTP, according to the manufacturer's recommendations. After labelling, the RNA was precipitated with ethanol and added directly to hybridization solution. A probe for the insertion sequence IS*6110* was prepared by the polymerase chain reaction (PCR) using chromosomal DNA as template and primers previously described (16). The probe was synthesized from 100 ng of *Mycobacterium tuberculosis* DNA (strain H37Rv) in 40 µl containing 1 unit of Taq polymerase in a buffer supplied by the manufacturer (Promega, USA), 1 µM primers, 100 µM dATP, 100 µM dCTP, 100 µM dGTP, 65 µM dTTP and 35 µM digoxigenin-dUTP (Boehringer Mannheim, Germany). DNA samples were initially heat-denaturated at 100 °C for 5 min, annealed at 68 °C for 1 min and extended at 72 °C for 1 min. Following the PCR, labelled DNA was purified by extraction with phenol/chloroform and precipitation with ethanol before heat denaturation for hybridization.

DNA Purification and Hybridization. Bacteria were grown in 50 ml cultures and the DNA extracted as previously described (17). Restriction enzyme digests of mycobacterial DNA were performed using about 1 µg of chromosomal DNA and 10 units of restriction enzyme for 5 h at 37 °C in incubation buffers supplied by the enzyme manufacturer (Boehringer Mannheim). The digested fragments were separated by electrophoresis through 24 cm, 0.7 % agarose gels at 70 V for 16 h. The DNA was transferred to positively charged nylon membranes under vacuum before hybridization and detection of the dioxigenin labelled probe performed as described previously (17).

Results

The four isolates of *Mycobacterium tuberculosis* showed progressive acquisition of drug resistance over time when studied by the proportion method using breakpoint concentrations (Table 1). Isolate A from March 1989 was resistant to isoniazid and rifampin, while isolate B from July 1989 was resistant to ethambutol and showed intermediate susceptibility to ansamycin (= rifabutin). The third isolate C from December 1989 was resistant to ansamycin, pyrazinamide and ofloxacin and the last isolate D from May 1990 was resistant to ciprofloxacin and sparfloxacin, two fluoroquinolones which were never used in this patient's treatment. Increased MICs of ofloxacin were also observed in the case of isolate C both using 7H11 agar and the Bactec radiometric method, and the finding of resistance also to ciprofloxacin and sparfloxacin was confirmed by these methods (Table 2). These findings were further confirmed by the viable colony counts as shown in Table 3, which suggested stepwise acquisition of resistance in the order ofloxacin > ciprofloxacin > sparfloxacin. Similar studies of the bactericidal effects of rifampin and ansamycin were not performed, however we did show stepwise acquisition of resistance to ansamycin as MICs in this case rose with each successive isolate in the order 0.5 µg/ml, 1.0 µg/ml, 2.0 µg/ml and > 2.0 µg/ml. It is noteworthy that ansamycin was not used in the therapeutic regimen of this patient, and the stepwise resistance to this rifamycin-related drug was a consequence of the use of rifampin during the first two months of therapy, not knowing that the initial isolate A was already resistant to it.

As shown in Table 4, the bacteriophage typing profiles of the four isolates were identical (Type A). The Southern blot hybridization with pTBN12 gave identical bands for all four isolates (Figure 1). Using the probe IS*6110*, the DNA band patterns were the same for isolates B, C and D, while isolate A showed a single band difference (Figure 2).

Discussion

The development of progressive drug resistance while undergoing treatment is of concern and the development of resistance to drugs which, while related, are considered to be more active than the

Table 2: MICs of three fluoroquinolones for the four isolates of *Mycobacterium tuberculosis* (A-D) measured using 7H11 agar medium and the Bactec radiometric method with 7H12 broth.

Isolate	MIC (µg/ml)					
	Ofloxacin		Ciprofloxacin		Sparfloxacin	
	7H11	Bactec	7H11	Bactec	7H11	Bactec
A	0.5	0.5	0.75	0.25	0.2	0.1
B	0.5	0.5	0.75	0.25	0.2	0.1
C	1.0	1.0	0.75	0.25	0.2	0.1
D	> 3.0	> 3.0	> 2.0	> 2.0	> 1.0	1.0

Table 3: Increase in resistance to three fluoroquinolones as shown by viable counts of three isolates of *Mycobacterium tuberculosis* from Bactec vials.

Experimental condition	Isolate		
	B	C	D
Control			
day 0	$9.9 \pm 0.9 \times 10^3$	$4.5 \pm 1.5 \times 10^3$	$4.3 \pm 0.7 \times 10^3$
day 7	$2.3 \pm 1.2 \times 10^5$	$1.0 \pm 0.5 \times 10^5$	$2.0 \pm 0.3 \times 10^6$
Ofloxacin			
0.5 µg/ml	$7.2 \pm 1.1 \times 10^3$	ND	ND
1.0 µg/ml	$4.5 \pm 0.7 \times 10^3$	$1.6 \pm 0.3 \times 10^4$	ND
2.0 µg/ml	ND	$2.9 \pm 0.9 \times 10^3$	ND
Ciprofloxacin			
0.5 µg/ml	$< 1.0 \times 10^1$	$5.0 \pm 0.6 \times 10^1$	ND
1.0 µg/ml	ND	$< 1.0 \times 10^1$	ND
Sparfloxacin			
0.1 µg/ml	$2.5 \pm 0.5 \times 10^2$	$1.8 \pm 1.2 \times 10^2$	ND
0.2 µg/ml	$9.5 \pm 0.5 \times 10^1$	$2.0 \pm 0.6 \times 10^1$	ND
0.5 µg/ml	$< 1.0 \times 10^1$	$< 1.0 \times 10^1$	ND
1.0 µg/ml	ND	ND	$5.0 \pm 1.0 \times 10^1$
1.5 µg/ml	ND	ND	$< 1.0 \times 10^1$

ND = Not done (only vials from MIC value onwards were titrated, and no screening was performed at concentrations above the Cmax values for the drugs in question).

Table 4: Mycobacteriophages used and phage typing profiles of the four isolates of *Mycobacterium tuberculosis*.

Mycobacterio-phage	Current designation[a]	Spot test dilution[b] (PFU/ml)	Host	Source	Lysis results[c]			
					Isolate A	Isolate B	Isolate C	Isolate D
DS6A	MPTH 2	10^6	*M. tuberculosis* H37Rv	W.B. Redmond Atlanta, USA	+/+	+/+	+/+	+/+
GS4E	MPTH 3	10^5	*M. tuberculosis* H37Rv	W.B. Redmond Atlanta, USA	–/–	–/–	–/–	–/–
BK1	MPTH 4	10^7	*M. smegmatis* ATCC 607	I. Baess, Copen-hagen, Denmark	–/–	–/–	–/–	–/–
BG1	MPTH 5	10^5	*M. tuberculosis* strain 1415	W.B. Redmond Atlanta, USA	–/–	–/–	–/–	–/–
D-34/14	MPTH 6	10^8	*M. tuberculosis* LR-14	S. Froman, Los Angeles, USA	–/–	–/–	–/–	–/–
DNA III 8	MPTH 7	10^7	*M. tuberculosis* H37Rv	E. Mankiewicz Montreal, Canada	–/–	–/–	–/–	–/–
PH	MPTH 9	10^6	*M. tuberculosis* H37Rv	K. Sushida, Tokyo Japan	–/–	–/–	–/–	–/–
SEDGE	MPTH 11	10^7	*M. smegmatis* ATCC 607	E. Mankiewicz Montreal, Canada	–/–	–/–	–/–	–/–
LEGENDRE	MPTH 12	10^8	*M. smegmatis* ATCC 607	E. Mankiewicz Montreal, Canada	–/–	–/–	–/–	–/–

[a] MPTH = Mycobacterial Phage Typing Human.
[b] Minimal concentration of virus in PFU/ml that gives almost confluent lysis on spotting 10 µl of stock on reference strains.
[c] Lysis results recorded on two independent screenings of the bacterial lawns; + complete lysis, – no lysis.

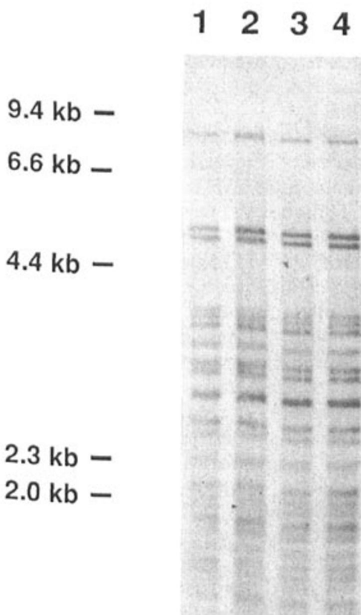

Figure 1: Southern blot of *Alu*I digested DNA from various *Mycobacterium tuberculosis* isolates probed with pTBN12. Lane 1, isolate D; lane 2, isolate C; lane 3, isolate B; lane 4, isolate A. The molecular weights of DNA markers are indicated in lane M.

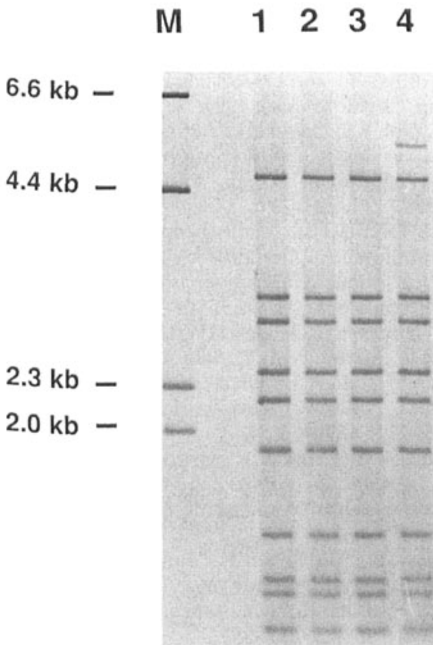

Figure 2: Southern blot of *Pvu*II digested DNA from various *Mycobacterium tuberculosis* isolates probed with IS*6110*. Lane 1, isolate D; lane 2, isolate C; lane 3, isolate B; lane 4, isolate A. The molecular weights of DNA markers are indicated in lane M.

drugs actually used, is disturbing. These findings required that the relatedness of all four isolates from this patient be determined to exclude the possibility of infection with multiple strains with differing drug susceptibilities.

We recently used bacteriophage typing to define five distinct lysotypes of *Mycobacterium tuberculosis,* namely A, AX, I, B and C, and have shown that lysotypes correlate with the geographic origin of patients (14, 15), phage type A being predominant among patients of Asian origin. Of 24 strains of *Mycobacterium tuberculosis* obtained from patients of Asian origin, 15 (63 %) were phage type A (unpublished data). Phage typing revealed that all four isolates in the present study were of phage type A (Table 4).

Although the stability of bacteriophage types of *Mycobacterium tuberculosis* during experimental murine infection has been established, this method is unfortunately limited in the range of types which can be demonstrated (14, 15). Genetic strain differentiation has greater potential to define sensitively differences between isolates if a genetic region with sufficient variability can be found. The insertion sequences IS*6110* and IS*986* are two variants of the same element which differ by only three nucleotide substitutions (18, 19). They have been used as probes in a number of epidemiologic studies (7, 20–22). The sequence is present in 1 to 19 copies per genome which are integrated into different sites (22). The newly described probe pTBN12 represents a repeated DNA element found in at least 30 copies per genome that is distinct from IS*6110* /IS*986* in several ways. Firstly, the restriction map of pTBN12 does not contain sites for *Pvu*II, *Bam*HI or *Xho*II, which are present in IS*6110* /IS*986* (8). Secondly, unlike the latter sequence, pTBN12 hybridizes to repeated elements in certain nontuberculous mycobacteria including *Mycobacterium szulgai, Mycobacterium kansasii* and *Mycobacterium gastri* (8). Detailed evaluation of pTBN12 is underway and information from a study of over 300 Australian case strains shows that strains can be separated into at least 200 types while showing relatedness for strains with geographic, social, family and other epidemiological links.

The single band difference seen with isolate A compared to the other isolates using IS*6110* as the probe is not unusual. Recent comparison of isolates from a cluster of 27 tuberculosis cases in homeless men and their associates in Australia gave identical band patterns in all isolates with

pTBN12 (data not shown). However IS*6110* showed single band differences in four cases. This suggests that band patterns are more stable with pTBN12 than with IS*6110*. A possible explanation is that one copy of the insertion element has been deleted from the chromosome in the interval between the isolation of isolates A and B. The relationship to the acquisition of drug resistance is not clear.

Because the results of both bacteriophage typing and RFLP typing were concordant for the isolates A, B, C and D, we regard this as conclusive of strain relatedness. The changes in drug susceptibility should not be attributed to mixed strain infection or multiple superinfection, rather they are due to changes occurring within a single infecting strain exposed to multiple antibiotics over time. The emergence of drug resistance, especially to new members of a class or new classes of drugs is alarming. In this case ofloxacin treatment resulted in resistance to this drug with the subsequent development of resistance to ciprofloxacin and sparfloxacin. We attribute the emergence of ansamycin resistance to the use of rifampin.

These findings are of concern in view of the upsurge in tuberculosis and other mycobacterioses in populations infected with HIV. The use of newer rifamycins, fluoroquinolones and macrolides for multiple drug resistant mycobacterial infections may prove to be more difficult than previously thought. Unsuccessful treatment of these infections with drugs from these classes may endanger the efficacy of new class members not yet released for clinical use. It is important that follow-up of cases with multiple drug resistance be instituted. Detailed studies of drug resistance mechanisms are now required. The careful development of management strategies for multiple drug resistant *Mycobacterium tuberculosis* infections must now be a priority.

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