Persister populations of *Mycobacterium tuberculosis* in sputum that grow in liquid but not on solid culture media

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Objectives: Can the characteristics of persisters in cultures of *Mycobacterium tuberculosis* also be found in bacilli from the sputum of pulmonary tuberculosis patients? The objective of this study was to explore whether the ability of persisters to grow in liquid but not on solid culture media, as in 100 day static cultures, can also be found in bacilli in sputum.

Methods: Serial dilutions of homogenized sputum obtained from patients before or during the first week of treatment were inoculated into broths to estimate the probable number of organisms and onto plates to give colony counts.

Results: Cultures in broths grew slowly to reach a maximal count at 12 weeks of probable numbers about 10-fold higher than the colony counts on plates, which did not grow beyond the initial count at 3–4 weeks. No such excess growth in liquid medium was found with control log-phase cultures.

Conclusions: About 90% of the bacilli in sputum are persisters that can grow in liquid media but not on solid plates.

Keywords: persister life cycle, therapy speed, persisters in sputum

Introduction

Cultures of *Mycobacterium tuberculosis* can be killed in days by concentrations of antibacterials such as rifampicin likely to be attained during therapy,[1] but the treatment of patients with regimens containing rifampicin as the main sterilizing drug takes 9 months to achieve relapse-free cure. The very slow rate of elimination of the bacilli in patients is usually attributed to the presence of persister bacilli, which may be tolerant to rifampicin and other antibacterials.[2,3]

The characteristics of persister bacilli have been described in static cultures held at 37°C in the depths of liquid culture medium at low O_2 tension. After about 3 weeks they pass through the non-replicating nrp1 and nrp2 stages, which are tolerant to high isoniazid concentrations but only marginally to rifampicin.[4] After 100 days, the cultures have developed populations that are tolerant to high concentrations of isoniazid and rifampicin and grow in liquid but not on solid culture media.[5,6] A further stage in development is the requirement for resuscitation promoting factor (Rpf), a low molecular weight protein produced by actively growing *M. tuberculosis*, for multiplication to start.[7]

It is reasonable to suppose that the air supply to tuberculous cavities may sometimes be sufficient to allow rapid aerobic growth of bacilli and at other times be cut off to create a low O_2 tension environment suitable for the creation of persisters. Sputum might therefore be supposed to contain persister populations at various stages of their development. The existence in sputum from patients with pulmonary tuberculosis of large numbers of an occult population requiring Rpf to multiply has recently been demonstrated,[8] but the earlier stages of persistence were not identified. We report here a study on the existence in sputum of bacilli that grow better in liquid medium than on solid medium, thus having the characteristics of an earlier stage in the life cycle of persisters than the requirement for Rpf.

Methods

Sputum was obtained before treatment from 14 smear-positive patients with pulmonary tuberculosis due to *M. tuberculosis* susceptible to isoniazid and rifampicin at King George V Hospital, Durban, South Africa and from a further 6 patients during the first week of their chemotherapy, when isoniazid might have eliminated multiplying bacteria.[9] The study was approved by the Medical Ethics Committees of the University of KwaZulu-Natal, Durban, South Africa and St George's Hospital Medical School, London, UK. Patients gave informed consent.

Specimens were sent by air to London; the interval between sputum collection and setting up the experiments was usually 2–3 days, during which

the sputum was either at low temperature during air transport or at 4°C in the London laboratory. The culture media (Becton, Dickinson, Oxford, UK) were Dubos base broth with albumin added and 7H11 agar with oleic acid, albumin, dextrose and catalase added. All media were made selective by the addition of 100 000 U/L polymyxin B, 10 mg/L trimethoprim, 100 mg/L ticarcillin and 10 mg/L amphotericin B (Mast Selectatab-Kirchner, Bootle, UK).[10] Sputum was homogenized by vortex mixing and incubation with an equal volume of 0.1% dithiothreitol (Sigma) for 30 min.

From this homogenate, serial 10-fold dilutions were made in distilled water from each of which 0.1 mL was spread on two sectors of selective 7H11 plates for colony counts. The number of colonies on the plates was counted after incubation for 3–4 weeks and the plates were then reincubated for at least another 4 weeks to see whether there was any increase in the colony count, with particular attention to plates with no colonies or only a few at the initial reading.

Serial 10-fold dilutions were also made in selective Dubos medium to create 13 sets of three 28 mL screw-capped bottles, each containing 10 mL of selective Dubos liquid medium. Each of the 39 liquid medium bottles was shaken and examined for visible growth of *M. tuberculosis* after incubation at 37°C for 4, 8 and 12 weeks. At the end of the 12 week incubation period, subcultures to 7H11 plates were made from the highest dilutions showing turbidity to demonstrate the typical colony morphology of *M. tuberculosis*, and also from bottles that did not show turbidity as these sometimes yielded growth on the plates. The probable number of organisms (pno) in the array of liquid medium cultures was calculated from statistical tables.[11]

Log-phase 7 day cultures were obtained from the sputum of seven patients and were examined as controls for growth in liquid and on solid media in the same way. The data were examined statistically using Stata (College Station, TX, USA).

Table 1. Comparison of colony counts from pre-treatment sputum on plates and pno in broth dilutions at 12 weeks with subcultivation

Duration of therapy (days)	Log_{10} viable count		Broth–plate, difference in counts
	plate (cfu/mL)	broth (pno/mL)	
0	7.52	8.24	0.718
0	9.45	9.24	−0.210
0	7.51	9.93	2.427
0	7.72	9.24	1.513
0	7.64	9.24	1.594
0	8.57	9.93	1.358
0	5.94	4.24	−1.703
0	8.48	8.93	0.455
0	5.46	8.24	2.775
0	6.90	7.58	0.674
0	5.97	6.58	0.609
0	7.06	8.93	1.867
0	7.79	8.93	1.139
Mean	7.395	8.370	1.017
SD			1.158
Upper 95% CI for the mean			1.717
Lower 95% CI for the mean			0.316

Results

The colony counts on plates and the final pno counts in liquid medium at 12 weeks with subcultivation from broth to plates are set out for the 14 sputa collected before treatment (Table 1), for the 6 sputa collected during the first week of treatment (Table 2) and for the 7 control log-phase cultures (Table 3), together with the calculated difference between broth and plate counts and the 95% CIs for these differences.

The means of the broth counts for all 20 sputa were higher than the plate counts by just over 1 log unit, showing that the excess bacterial population comprised about 90% of the total population, with the CIs for the difference lying well above zero in Table 1. By

Table 2. Comparison of colony counts on plates and pno in broth dilutions at 12 weeks with subcultivation from sputum collected during the first week of treatment

Duration of therapy (days)	Log_{10} viable count		Broth–plate, difference in counts
	plate (cfu/mL)	broth (pno/mL)	
2	6.71	7.24	0.53
2	6.19	10.24	4.05
2	5.92	5.93	0.01
2	6.83	8.24	1.41
2	6.83	6.58	−0.26
7	4.62	5.93	1.31
Mean	6.183	7.360	1.175
SD			1.56
Upper 95% CI for the mean			2.81
Lower 95% CI for the mean			−0.46

Table 3. Comparison of colony counts on plates and pno in broth dilutions at 12 weeks with subcultivation on log-phase cultures

	Log_{10} viable count		Broth−plate, difference in counts
	plate (cfu/mL)	broth (pno/mL)	
	8.77	9.24	0.47
	8.56	8.58	0.02
	8.55	7.93	−0.62
	8.86	8.58	−0.29
	8.92	8.58	−0.35
	9.08	9.93	0.85
	8.82	7.93	−0.89
Mean	8.796	8.681	−0.115
SD			0.56
Upper 95% CI for the mean			0.45
Lower 95% CI for the mean			−0.68

Table 4. Counts of *M. tuberculosis* colonies on plates as $\log_{10}$ cfu, and as estimates of pno in broth, with readings at 4, 8 and 12 weeks and on subculture to plates at 12 weeks

	Broth, 4 weeks	Broth, 8 weeks	Broth, 12 weeks	Broth cultures incubated for 12 weeks and then subcultured to plates	Plate count
Sputum specimens ($n=20$)					
mean	6.281	6.880	7.365	8.066	7.032
SEM	0.441	0.348	0.334	0.353	0.257
upper 95% CI for the mean	7.20	7.61	8.07	8.804	7.57
lower 95% CI for the mean	5.36	6.15	6.67	7.328	6.49
Log-phase cultures ($n=7$)					
mean	6.818	7.717	8.104	8.681	8.796
SEM	0.630	0.404	0.357	0.269	0.072
upper 95% CI for the mean	8.36	8.71	8.98	9.34	8.97
lower 95% CI for the mean	5.28	6.73	7.23	8.02	8.62

contrast, the means of the broth counts for the control log-phase cultures (Table 3) were all slightly lower than the plate counts throughout. The full readings of the broths at all the timepoints and the plate counts are summarized in Table 4.

The pno from sputa gradually rose over the 12 week incubation period to 7.365 log cfu/mL at 12 weeks, higher than the plate count of 7.032 log cfu/mL, but only significantly exceeded the plate counts when broths were read at 12 weeks and subcultures made to plates, showing that growth in some of the broths was so slow as to be insufficient to produce visible turbidity but was still capable of subcultivation to plates. The broth counts showed a gradual increase in pno during the 12 week incubation period, while the colony counts on solid medium (including plates with a few or no colonies at 3–4 weeks) showed no increase following the initial readings. Thus there seemed to be a majority bacterial population that grew very slowly in liquid medium during prolonged incubation over at least a 12 week period but did not grow on solid medium.

Control log-phase cultures also showed a gradual increase in pno in liquid medium during the 12 week incubation period, but the means of the broth counts, even those incubated for 12 weeks with subcultivation, were never greater than the mean plate colony counts. Thus the bacterial population that grew in liquid but not on solid medium was not found in the log-phase cultures. Individual counts on sputa showed considerable variability in the difference between liquid and solid medium counts, ranging from negative differences in three sputum specimens to a maximum of $\log_{10}$ 4.047 in one of the 20 results (Tables 1 and 2). The results from the 14 pre-treatment sputum specimens and the 6 obtained during the first week of treatment appeared similar, though the high variability of the results from these 6 specimens may have hidden a trend.

Discussion

The large bacterial population in sputum that can grow in liquid media, albeit very slowly, but not on solid medium plates seems to correspond to the component in the Hu/Coates models that is found when static liquid medium cultures have been incubated undisturbed for 100 days under microaerophilic conditions.[5,6] This persister component was characterized by an ability to grow in liquid medium but not on solid medium, though once it started to grow in liquid medium it rapidly recovered an ability to grow on solid medium, as did our subcultures from broths at 12 weeks.

This component comprised an increasing proportion of the total population during the incubation, but at 100 days it consisted of only about 10^3 bacilli/mL out of a total of $\sim 10^7$ bacilli/mL. By contrast, our persister population predominated to form some 90% of the sputum population after incubation for 12 weeks, suggesting that even longer periods of incubation might be necessary to reveal the true size of a population. This bacterial population was apparently not encountered in the work on the Rpf-requiring stage in sputum.[8] Reasons for this might be the use of NaOH (known to be bactericidal to *M. tuberculosis*) to decontaminate the sputum specimens rather than our use of selective medium, or the long period of incubation necessary to demonstrate the population.

The long incubation period of 12 weeks found necessary for the liquid medium cultures to attain their maximal bacillary count shows that sputum cultures in liquid medium should also be cultured for similarly lengthy periods before they are considered negative, as has been suggested previously.[12] *M. tuberculosis* that grew better in liquid media than on solid plates and were thought to be persisters have also been obtained from murine infections of 10 months duration.[13]

Acknowledgements

We wish to thank Dr Roxana Rustomjee, former Director of the TB Unit of the South African Medical Research Council in Durban, and Ms Jenny Allen of the same unit, who enabled and oversaw the collection and preparation of specimens in Durban for shipment to St George's.

Funding

Sputum specimens were examined as part of the quality assurance programme of the Oflotub clinical trial,[14] financially supported by a grant (ICA4-CT2002-10057) from the European Commission and another

(OD/TS-04-00291) from the WHO special programme for research and training in tropical diseases (TDR).

Transparency declarations

None to declare.

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