Resuscitation-promoting Factors Reveal an Occult Population of Tubercle Bacilli in Sputum

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Rationale: Resuscitation-promoting factors (Rpfs) are a family of secreted proteins produced by *Mycobacterium tuberculosis* (Mtb) that stimulate mycobacterial growth. Although mouse infection studies show that they support bacterial survival and disease reactivation, it is currently unknown whether Rpfs influence human infection. We hypothesized that tuberculous sputum might include a population of Rpf-dependent Mtb cells.

Objectives: To determine whether Rpf-dependent Mtb cells are present in human sputum and explore the impact of chemotherapy on this population.

Methods: In tuberculous sputum samples we compared the number of cells detected by conventional agar colony-forming assay with that determined by limiting dilution, most-probable number assay in the presence or absence of Rpf preparations.

Measurements and Main Results: In 20 of 25 prechemotherapy samples from separate patients, 80–99.99% of the cells demonstrated by cultivation could be detected only with Rpf stimulation. Mtb cells with this phenotype were not generated on specimen storage or by inoculating sputum samples with a selection of clinical isolates; moreover, Rpf dependency was lost after primary isolation. During chemotherapy, the proportion of Rpf-dependent cells was found to increase relative to the surviving colony-forming population.

Conclusions: Smear-positive sputum samples are dominated by a population of Mtb cells that can be grown only in the presence of Rpfs. These intriguing proteins are therefore relevant to human infection. The Rpf-dependent population is invisible to conventional culture and is progressively enhanced in relative terms during chemotherapy, indicating a form of phenotypic resistance that may be significant for both chemotherapy and transmission.

Keywords: tuberculosis; resuscitation-promoting factors; sputum; non-replicating cells

Tuberculosis remains a major killer and a great challenge to global health. With an estimated 9 million new cases and 2 million deaths annually the extraordinary ability of *Mycobacterium tuberculosis* (Mtb) to subvert the immune system and defy our control efforts through chemotherapy is evident (1, 2). Nonreplicating cells of Mtb are widely believed to contribute to the burden of disease by underpinning both latent infection and the slow response to chemotherapy (3); moreover, the likely presence of nonreplicating cells was recently demonstrated in a study of human tuberculous sputum samples (4). Despite these respectively indirect and direct lines of evidence of nonreplicating bacillary populations arising

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The resuscitation-promoting factors are a family of secreted proteins produced by *Mycobacterium tuberculosis*. They act on the bacterial cell wall, stimulate regrowth of inert and otherwise nonculturable bacteria, and enable reactivation of chronic tuberculosis in mice but have no established role in human infection.

What This Study Adds to the Field

Most tuberculous sputum samples are dominated by a population of *M. tuberculosis* cells that can be cultured only by addition of resuscitation promoting factors. During chemotherapy this occult population increases relative to the bacilli that can be recovered by conventional culture.

during human infection, the factors responsible for the subsequent reactivation of Mtb growth that must take place for spread of infection and disease progression are presently unknown.

Resuscitation-promoting factors (Rpfs) are the first example of mycobacterial proteins playing an important role in reactivation of chronic infection (5–7). Originally discovered as proteins stimulating mycobacterial growth and regrowth of nonreplicating cells obtained *in vitro* (8–10), they were later demonstrated to be important for *in vivo* persistence in mice (11, 12) and one study has provided preliminary evidence of an effect on mycobacteria in clinical samples (13). Although the precise mechanism by which Rpfs effect their growth-stimulatory action remains unknown, they possess an enzyme activity related to lysozyme, which hydrolyses bacterial cell walls and likely belongs to the family of lytic transglycosylases (14–17). Site-directed mutagenesis studies have revealed that the Rpf enzyme activity is indispensable for the resuscitation and growth-stimulatory effects of these proteins (15).

Although the importance of Rpfs has been established for Mtb infection in mice (11, 12), evidence of a role for these proteins in human tuberculosis is currently lacking. In light of the evidence we have previously presented for nonreplicating tubercle bacilli in sputum (4), we hypothesized that there might be Rpf-dependent cells present in these samples. Here we report our findings on the influence of various Rpf preparations on the cell numbers recovered by culture from sputum samples taken from patients with pulmonary tuberculosis before and shortly after starting chemotherapy.

Some of the results of these studies have been previously reported in the form of abstracts (18, 19).

METHODS

Patients

The study was approved by Leicestershire, Northamptonshire, and Rutland Research Ethics Committee (07/Q2501/58). Patients were identified as sputum smear positive by routine microscopy of auramine-

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stained samples and, after informed consent had been obtained, agreed to provide sputum samples for characterization of mycobacterial populations before and after onset of chemotherapy. All patients were microbiologically confirmed to have tuberculosis; in many cases early confirmation was obtained by application of an in-house real-time mycobacterial polymerase chain reaction (20).

Bacterial Strains

The type strain of *M. tuberculosis*, H37Rv, was used to produce Rpf-containing supernatants and in many of the experiments subsequently described. A strain derived from Mtb H37Rv in which all five Rpf genes had been deleted (12) was kindly provided by V. Mizrahi (University of Witwatersrand, Johannesburg, South Africa). Additional isolates were obtained from the samples processed in this study.

Sputum Decontamination and Growth Assays

Sputum samples were stored at 4°C on receipt. Most were analyzed within 2 days (the practice in many diagnostic laboratories) and a few were stored for up to 10 days. Samples subjected to growth assays were assigned numbers 1–25 and these designations were used throughout. Freezing of sputum samples resulted in a significant loss of cell culturability both on agar plates and in liquid medium and such samples were excluded from the main analysis but were used where indicated in some *in vitro* experiments. All sputum samples were decontaminated before growth analyses by mixing 0.5 ml of sputum with 0.5 ml of 4% (wt/vol) NaOH and incubating for 15 minutes at room temperature followed by neutralization with 1 ml of 14% (wt/vol) KH₂PO₄. Decontaminated samples were centrifuged at 2,000 × *g* for 15 minutes and resuspended in 2 ml of 7H9 medium, and appropriate samples were used for growth assays. All growth media were supplemented with the antibiotic mixture PANTA (polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; BD Biosciences, San Jose, CA) as recommended by the manufacturer.

Mtb cells from sputum samples were grown in 7H9 liquid supplemented with 10% (vol/vol) OADC (oleic acid, albumin, dextrose, and catalase; BD Biosciences), 0.05% (wt/vol) Tween 80 or on 7H10 agar plates. For resuscitation, medium was supplemented with a 20 nM concentration of either recombinant RpfE or RpfB (9) or with 50% (vol/vol) sterile culture supernatant prepared as previously described (10). Briefly, Mtb cells were grown in the supplemented 7H9 medium in roller bottles to mid-exponential stage (OD_{600 nm}, 0.6–0.9). After centrifugation supernatants were filtered by means of vacuum filtration system units (VWR, West Chester, PA) and applied for experiments immediately. Sterility of the culture supernatant was checked in each experiment. Most probable number (MPN) assays were performed in quadruplicate replicates in 48-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany), by diluting 50 µl of cell suspension in 450 µl of the medium; MPN counts were calculated with 95% confidence limits according to the U.S. Food and Drug Administration procedure (21). Where MPNs were performed with culture supernatant supplementation these were designated MPN_Rpf+SN or MPN_Rpf–SN as appropriate (the latter denoting supernatant from the Rpf deletion mutant). Growth was confirmed as mycobacterial by auramine staining. In addition, randomly chosen samples grown with or without Rpf or culture supernatant were checked for consistency with the patient's original isolate by MIRU-VNTR (mycobacterial interspersed repetitive units–variable number tandem repeats) typing (22). For colony-forming unit (CFU) assays sputum samples were serially diluted in supplemented 7H9 medium and 10 µl of each dilution was placed on a 7H10 agar plate. All dilutions were made in quadruplicate. Plates were incubated for up to 8 weeks and colonies from appropriate dilutions were counted.

We have used two indices to signify different effects observed in sputum samples. They are defined as follows: resuscitation index (RI) = $\log_{10}(\text{MPN_Rpf+SN/CFU})$; inhibition index (II) = $\log_{10}(\text{MPN/CFU})$.

Detection of Growth-inhibitory Activity of Sputum Samples *in Vitro*

To replicate as closely as possible the effect of tuberculous sputum on Mtb, we studied the culture properties of Mtb H37Rv cells inoculated into previously frozen tuberculosis sputum samples. Weakly culture-positive sputum samples were rendered culture negative by freezing, before inoculation of Mtb H37Rv cells. Mtb H37Rv cells (5×10^5 cells/ml),

grown to mid-exponential phase, were inoculated into two different sputum samples (rendered culture negative by freezing) and incubated for 24 hours at 4°C. In control samples, cells were incubated at the same density in phosphate-buffered saline.

Effect of Storage of Sputum at 4°C on Mtb Populations

A single sputum sample (from patient 25) was received and processed within 2 hours of expectoration. The sample was mixed carefully with a serological pipette to achieve as uniform distribution of the Mtb content as possible and then divided into 0.5-ml aliquots. One aliquot was immediately processed and assayed as described previously and the remainder were stored at 4°C for the periods indicated before assay.

In a separate experiment a pathogen-free sample of sputum was prepared by pooling the residue from previously analyzed samples (stored at 4°C) from several patients with chronic obstructive pulmonary disease. Aliquots of this pooled sample were then separately contaminated with Mtb strains and subjected to serial assay as indicated previously. The sputum samples were carefully mixed together with a serological pipette and separated into 5-ml aliquots. Cells (5×10^5 cells/ml) of Mtb H37Rv and nine clinical Mtb isolates grown to mid-exponential phase were inoculated into separate aliquots of the pooled TB-negative sputum, mixed well with a serological pipette, and stored at 4°C for the periods indicated before sampling and processing as described previously.

Direct Rifampin Treatment of Mtb Cells in Sputum

Three sputum samples from separate patients were decontaminated as described previously and then washed by incubation in 7H9 medium containing 0.05% (wt/vol) Tween 80 and PANTA supplement for 14 hours and centrifuged before exposure to rifampin. Pre- and postwashing CFU were indistinguishable. In preliminary experiments we had found that exposure to antibiotic without prior washing led to negative cultures. Washing led to the results presented and we suggest this may be due to removal of the sputum inhibitory activity. The pellets from 0.5 ml of original sputum were resuspended in 10 ml of 7H9 medium supplemented with OADC (10%, vol/vol), 0.05% (wt/vol) Tween 80, PANTA supplement, and rifampin (1 µg/ml). After incubation for 7 days cultures were centrifuged and resuspended in 10 ml of fresh medium, and growth assays were performed.

RESULTS

Growth Assays Reveal Rpf-dependent Cells as the Dominant Population of Mtb in Most Samples of Tuberculous Sputum

We studied prechemotherapy microscopy–positive human sputum samples, an accessible source of tubercle bacilli adapted *in vivo*, and compared the number of cells detected by standard agar CFU assay with that determined by limiting dilution, MPN assay in liquid medium in the presence or absence of various Rpf preparations. In initial experiments we found that most samples contained an excess of cells that could be demonstrated only by addition of recombinant RpfE (Figure 1) or RpfB (data not shown). However, fresh culture supernatant, which we have previously observed to contain high levels of Rpf activity, was consistently the most potent in this regard (Figure 1; and *see* Table E1 in the online supplement). Availability of an Mtb strain in which all five Rpf-encoding genes have been deleted (12) enabled us to conduct appropriate controls and isolate effects due to Rpf activity. Resuscitation activity of the Rpf-free culture supernatants was measured in seven sputum samples from separate patients (Figure 2). In all cases this control culture supernatant did not show significant stimulation above the CFU count, thereby providing solid evidence of the central role of Rpf in revealing what we have identified as the Rpf-dependent population of Mtb in sputum. Therefore all further studies described subsequently were performed with culture supernatant as a source of Rpfs.

In 20 of 25 prechemotherapy samples from separate patients, at least 80% of the cells demonstrated by culture could be

Figure 1. Resuscitation-promoting factor E (RpfE) and culture supernatant stimulate growth of *Mycobacterium tuberculosis* (Mtb) cells undetectable by conventional methods from sputa. The most probable number (MPN) count obtained in 7H9 medium (gray columns) or the same medium supplemented either with Rpf-containing culture supernatant (Rpf+SN; open columns) or recombinant RpfE (hatched columns) is compared with the cell count determined by colony-forming unit (CFU) assay (solid columns). Error bars indicate 95% confidence limits for MPN counts and standard deviation values for CFU.

detected only with Rpf stimulation (Table 1 and see Table E1). In other words, the Rpf-dependent population exceeded the cell count obtained by conventional methods (CFU or MPN) by at least fourfold. In three samples (patients 10, 18, and 21) Rpf-dependent cells comprised about 65% of the population; no Rpf response was observed in the remaining two samples (patients 8 and 24). The sample content of Rpf-dependent cells, which cannot be detected by any other culture technique, is most simply revealed by what we have called the resuscitation index (RI). This is obtained from the Rpf-supplemented MPN count divided by the cognate CFU count and expressed as $\log_{10}$. We selected CFU rather than MPN without Rpf as the denominator for the RI because, in most cases, the latter yielded counts lower

Figure 2. Effect of resuscitation-promoting factor (Rpf)-free supernatants on resuscitation of *Mycobacterium tuberculosis* (Mtb) cells from sputum. Rpf-free culture supernatant was isolated from the quintuple Rpf deletion Mtb mutant (Rpf-SN). The resuscitation index (RI) was calculated as the ratio of the various most probable number (MPN) counts obtained to the colony-forming unit (CFU) counts. Asterisks indicate samples in which the RI exceeds 1 (in each case the lower 95% confidence interval for the RI was >0.48). The various MPN counts were obtained in 7H9 liquid medium with the addition of Rpf-containing culture supernatant (Rpf+SN) (open columns) or Rpf-free culture supernatant (Rpf-SN) (solid columns). Error bars indicate 95% confidence limits for MPN counts.

TABLE 1. RESUSCITATION-PROMOTING FACTOR-DEPENDENT POPULATIONS IN PRETREATMENT TUBERCULOSIS SPUTUM SAMPLES FROM 25 SEPARATE PATIENTS

Patient	Log CFU	Log MPN*	Log MPN Rpf+SN†	Resuscitation Index	Inhibition Index	Rpf-dependent Population (%)‡
1	2.00	2.64	5.42	3.42	0.64	99.83
2	4.43	4.28	5.28	0.75	-0.15	85.90
3	3.90	2.26	5.11	1.21	-1.64	93.76
4	4.89	2.26	6.48	1.58	-2.73	97.44
5	3.90	3.26	5.28	1.38	-0.64	95.83
6	4.07	3.88	5.10	1.03	-0.19	90.70
7	2.90	3.28	5.30	2.40	0.38	99.05
8	5.26	3.11	5.11	-0.15	-2.15	N/D
9	5.41	5.17	6.38	0.97	-0.24	89.10
10	6.00	6.28	6.75	0.75	0.28	65.71
11	4.30	4.28	5.28	0.98	-0.02	89.60
12	4.74	3.28	6.11	1.37	-1.46	95.70
13	2.00	2.48	5.42	3.42	0.48	99.88
14	4.72	3.48	7.05	2.33	-1.24	99.53
15	5.75	2.45	7.05	1.30	-3.30	95.00
16	3.60	3.26	6.75	3.15	-0.34	99.93
17	4.95	2.26	7.05	2.10	-2.69	99.20
18	5.12	5.64	6.11	1.01	0.52	65.63
19	<1	<2	4.66	>2.66	N/A	99.98
20	6.26	3.16	7.05	0.79	-3.10	83.93
21	6.60	4.04	7.05	0.45	-2.56	66.70
22	5.80	6.05	7.05	1.25	0.25	90.00
23	6.83	7.08	8.08	1.25	0.25	90.00
24	5.30	6.11	6.11	0.81	0.81	N/D
25	5.86	5.28	6.78	0.92	-0.58	88.15

Definition of abbreviations: CFU = colony-forming units; MPN = most probable number; N/A = not applicable; N/D = not detected; Rpf = resuscitation-promoting factor.

* Values are quoted without confidence intervals for greater clarity. The complete data set is given in Table E1 in the online supplement.

† MPN Rpf+SN denotes the MPN counts obtained in the presence of Rpf-containing supernatant or recombinant RpfE (samples 2, 3, 11, and 14).

‡ The Rpf-dependent population reflects the excess of cells that could be detected only by Rpf supplementation (i.e., neither MPN nor CFU would detect these cells). In most samples the CFU gave the next highest count after the MPN/SN count, but in samples 1, 7, 10, 13, 18, 22, 23, and 24 the MPN was higher than the CFU. In each case the percentage values were calculated with respect to the next highest count to the MPN_Rpf+SN, thus identifying the Rpf-dependent population. Boldface type denotes samples in which the Rpf-dependent cell count was significantly greater than the next highest count as judged by the 95% confidence intervals for the MPN/SN determination. Resuscitation index (RI) = $\log_{10}(\text{MPN_Rpf+SN}/\text{CFU})$; inhibition index (II) = $\log_{10}(\text{MPN}/\text{CFU})$; Rpf-dependent population = $[(\text{MPN_Rpf+SN} - \text{CFU}) \div \text{MPN_Rpf+SN}] \times 100$.

than the CFU and because we needed an operational standard point of reference. As is evident from the results presented in Table 1 and Table E1, sputum samples exhibit a complex combination of influences on the growth of Mtb cells. We focus here on the numerically dominant effects, although others clearly merit further investigation. We note in particular that sputum often contains an inhibitory activity for mycobacterial growth in liquid medium and this is characterized further subsequently.

Inhibitory Activity against Mtb Growth in Liquid Medium Is Present in Sputum

The tendency for unsupplemented MPN counts to be lower than CFU counts is identified in Table 1 by derivation of an inhibitory index (II; $\log_{10} \text{MPN}/\text{CFU}$). Effects in excess of 10-fold were common and exceeded 1,000-fold in 2 of 25 samples. Inclusion of additional washing steps applied to the decontaminated cultures before dilution resulted in higher MPN counts but had no effect on the CFU count (data not shown). This washing effect is consistent with partial removal of a cell-bound inhibitory activity because the effect is preserved at high dilution in the MPN assay.

Figure 3. Inhibitory activity of sputum samples on the growth of *Mycobacterium tuberculosis* (Mtb) H37Rv cells in liquid medium. Mid-log-phase Mtb cells were spiked into two different culture-negative sputum samples from patients with tuberculosis and were incubated for 24 hours at 4°C. Control cells were kept in phosphate-buffered saline for 24 hours at 4°C. *Solid columns*, colony-forming units (CFU); *gray columns*, most probable number (MPN) in 7H9 medium; *open columns*, MPN in the presence of Rpf-containing culture supernatant (Rpf+SN).

To determine whether this activity could be detected directly in sputum samples, laboratory cultures of Mtb H37Rv were inoculated into samples previously rendered culture negative by freezing. Figure 3 shows that substantial inhibitory activity was present in the two samples tested. Interestingly, although addition of Rpf-containing supernatant overcame this inhibition, incubation of Mtb H37Rv in sputum did not lead to the development of an Rpf-dependent population.

Storage of Sputum Does Not Induce Rpf Dependency

Because some of our sputum samples were stored for several days before analysis it was important to assess whether storage led to the development of Rpf dependency. We first analyzed our results to determine whether the levels of Rpf-dependent cells correlated with storage time. Using the data presented in Table E1, we found no such correlation (r^2 for storage time vs. RI, 0.0083). We next processed an Mtb-positive sputum sample at various times after expectoration. No significant change in the Rpf-dependent population was observed over 10 days of storage (Figure 4).

We next addressed the possibility that differences between infecting Mtb strains might lead to differences in the development of Rpf dependency during storage. We investigated nine of the isolates from the various patients studied here; five of these had been associated with high Rpf-dependent MPN count cells in the original sputum sample and four had not produced such cells. Mtb H37Rv was applied as a control strain. None of the nine clinical isolates produced detectable, statistically significant populations of Rpf-dependent cells during early or late exponential phase growth (data not shown) or after inoculation into a pooled pathogen-free sputum sample followed by sampling over 10 days (*see* Table E2 in the online supplement).

The Rpf-dependent Population in Sputum Is Enhanced Relative to the Colony-forming Population during Chemotherapy

To monitor the response of Rpf-dependent cells to chemotherapy, samples from eight patients taken 7–11 days after starting chemotherapy were studied and the decline in cell counts from pretreatment samples was determined. Figure 5 shows that declines in CFU counts correlated well ($r^2 = 0.879$) with those observed by Rpf-supplemented MPN assay. However, in all

Figure 4. Storage of a freshly expectorated tuberculous sputum sample does not produce additional resuscitation-promoting factor (Rpf)-dependent *Mycobacterium tuberculosis* (Mtb) cells. Sputum from patient 25 was assessed for colony-forming units (CFU), most probable number (MPN), and MPN_Rpf+SN counts immediately after collection (within 3 h) and at the times shown after storage at 4°C. *Solid columns*, CFU; *gray columns*, MPN in 7H9 medium; *open columns*, MPN in the presence of Rpf-containing culture supernatant (Rpf+SN).

samples the CFU-detected cells decreased to a greater extent than the Rpf-dependent populations (Figure 5). A simple interpretation of the linear regression equation ($\log_{10} \text{MPN Rpf+SN} = 1.305 \times \log_{10} \text{CFU} - 1.078$) indicates that CFUs decrease 10-fold before any effect is seen on the Rpf-dependent population and that above this threshold colony counts are affected at 20-fold over the supplemented MPN counts.

The Rpf-dependent and CFU-producing populations in sputum were monitored for longer periods of treatment in four patients (Table 2). In all but one case the RI continued to increase after the initial period covered by Figure 5. In three samples no growth was obtained by CFU or unsupplemented MPN. In these cases up to 10^4 cells capable of replication were evidently present yet undetectable by conventional culture. The results for patient 24 are noteworthy because no Rpf-stimulated cells were detected in the initial pretreatment sample or in subsequent samples up to 14 days (MPN counts with and without Rpf were indistinguishable).

Figure 5. Chemotherapy induces a decline in resuscitation-promoting factor (Rpf)-dependent cells that is correlated with but slower than the accompanying fall in colony-forming unit (CFU) counts. Changes in CFU and most probable number (MPN) counts were calculated as the $\log_{10}$ ratio of CFU or Rpf-supplemented MPN count before the onset of treatment to the corresponding count during treatment. Treatment sputum samples were collected 7–11 days after the start of chemotherapy.

TABLE 2. RESUSCITATION-PROMOTING FACTOR-DEPENDENT POPULATIONS IN POSTTREATMENT TUBERCULOSIS SPUTA

Patient	Treatment Day	Microscopy	Log CFU	Log MPN	Log MPN Rpf+SN	RI*
20	27	N/D	<1 [†]	2.26	3.48	>2.48
	43	N/D	<1 [†]	<2	5.22	>4.22
21	31	Scanty	<1 [†]	<2 [‡]	5.42	>4.42
23	115	Scanty	<1 [†]	<2 [‡]	5.11	>4.11
	116	Scanty	<1 [†]	4.28	6.25	>5.25
24	14	Negative	<1 [†]	1.31	1.31	>0.31

Definition of abbreviation: RI = resuscitation index.

[†] Limit of detection, no colonies observed.

[‡] Note that routine mycobacterial growth indicator tube (MGIT) and Lowenstein-Jensen (LJ) diagnostic culture were negative in these cases.

* For all patients except patient 24 (see RESULTS) the RI exceeded the previous sample value by at least 1 (see Table 1).

Direct Treatment of Sputum Demonstrates That the Rpf-dependent Population in Sputum Is Rifampin Tolerant

Three decontaminated microscopy-positive sputum samples from separate patients were incubated in the presence or absence of rifampin for 1 week and the CFU and MPN counts were determined (Figure 6). CFU counts fell below the limit of detection in all cases after 7 days of exposure to rifampin whereas the Rpf-stimulated population remained essentially unchanged. A parallel control with Mtb H37Rv showed that when the inoculum contained no Rpf-dependent cells substantial declines occurred in the supplemented MPN count.

DISCUSSION

By application of simple growth assays we demonstrated that Mtb populations in most microscopy-positive clinical sputum samples are dominated by cells whose growth is dependent on Rpf proteins. These proteins possess cell wall-hydrolytic activity and are widely distributed among the actinobacteria (8, 9). Rpf-dependent cells cannot resume normal growth unless they are resuscitated and therefore remain “invisible” to standard culture. Because after propagation *in vitro* Rpf dependency is lost, we conclude that this property results from a phenotypic rather than a genetic change in Mtb.

We first showed (Figure 1) that Mtb culture supernatants had superior growth-stimulatory activity relative to purified recombinant Rpf and that this activity was absent in supernatants from an Mtb strain in which all five Rpf-encoding genes had been deleted (Figure 2). Wild-type Mtb culture supernatants contain a mixture of the Rpf, which may account for this improved potency. In addition, it is notoriously difficult to stabilize the biological activity of purified Rpf (15, 16) and the supernatants used here remain the most convenient and reliable source with which to effect growth stimulation. Although the use of this unpurified material raises concerns regarding the specificity of the effects, the exceptional control provided by the deletion mutant compensates for this. We cannot formally exclude the influence of other proteins (e.g., RipA [17] or other peptidoglycan hydrolases) in our experiments. Nonetheless, the specific effects of Rpf remain the simplest explanation of our resuscitation results.

In addition to revealing previously undetected viable Mtb cells, the growth assays applied here have demonstrated differences between patients in terms of the distributions between the various bacillary populations detected. Thus, although most samples were dominated by Rpf-dependent cells, some were not. Moreover, many sputa were shown to have variable amounts of an apparently cell-associated inhibitory activity. The nature of

Figure 6. Effect of rifampin treatment *in vitro* on viability of *Mycobacterium tuberculosis* (Mtb) cells in sputum. Decontaminated sputum samples were incubated in 7H9 medium containing PANTA antibiotic supplement for 14 hours, and then treated with rifampin (1 μ g/ml) in 7H9 medium containing PANTA for 7 days at 37°C with shaking. Solid columns, colony-forming units (CFU) before rifampin treatment; gray columns, CFU after 7 days of treatment (note: these were all negative, i.e., CFU < 10/ml); open columns, MPN in Rpf+SN before treatment; hatched columns, MPN in Rpf+SN after treatment. Error bars indicate 95% confidence limits for MPN counts and standard deviation values for CFU.

this activity is presently unknown. The description of a secreted leukocyte protease inhibitor on the surface of Mtb cells in sputum offers one potential explanation (23). Although further studies will be required to establish the clinical significance of these differences, our additional and related studies allow some initial consideration of the possibilities.

We considered the possibility that Rpf dependency might occur in response to storage of sputum samples. Analysis of the storage time of our samples and the cognate Rpf-dependency counts did not reveal any correlation. Furthermore, serial assay of one freshly expectorated sample from 3 hours to 10 days after expectoration failed to reveal any significant change in the Rpf-dependent population. Finally, to address the possibility that strain differences might have affected our results, we studied nine different clinical Mtb isolates that had been associated with a wide range of Rpf-dependent counts in the sputum samples from which they were isolated. These isolates were tested for production of Rpf-dependent cells both during growth in broth and as a result of inoculation into a pooled pathogen-free sputum sample and storage at 4°C for 10 days. Rpf-dependent cells were not produced in any of these studies. We conclude that there is no evidence that Rpf dependency develops during sample storage and that it is not an inherent feature of particular clinical strains. Rather, it appears that exposure to certain conditions *in vivo* before expectoration elicits Rpf dependency. We note that mouse intraperitoneal inoculation of Mtb and subsequent recovery from peritoneal macrophages has previously been reported to result in Rpf dependency (24).

The changes we observed in the Mtb populations in sputum during early chemotherapy reveal that the Rpf-dependent population becomes more prominent as CFU counts decline. A simple interpretation of the relationship shown in Figure 5 suggested that Rpf-dependent cells are eliminated at approximately one-twentieth the rate of their colony-forming counterparts. However, we emphasize that this quantitative interpretation is based on a small number of samples and further studies are required. Nonetheless, it appears that either an Rpf-dependent population present at the time of initiating chemotherapy is eliminated more

slowly than the colony-forming population, or Rpf-dependent cells are induced by chemotherapy.

Our preliminary study on the bactericidal effect of rifampin on the Rpf-dependent Mtb population in sputum failed to show any decline in this population over 7 days. In the same samples both the CFU count and the unsupplemented MPN count fell to below their respective limits of detection. Thus the Rpf-dependent cells in these samples were tolerant to rifampin whereas their conventionally culturable counterparts were not. Rifampin tolerance has been demonstrated in a number of previous studies (4, 25–27) and is generally associated with conditions in which the target Mtb cells were not replicating. Thus the presence of the Rpf-dependent population, its relative preservation during chemotherapy, and our evidence of its rifampin tolerance all add further weight to the view that sputum frequently contains a population of nonreplicating Mtb cells.

CFU-based studies of sputum samples during the early phase of chemotherapy have been used to evaluate responses to different chemotherapeutic regimens and have been formalized as early bactericidal activity (EBA) analyses (28). We emphasize that in the present study we have not followed the defined protocols required for an EBA study; nonetheless, the relationships between the populations we have demonstrated and EBA results are worthy of consideration. The relative preservation of the Rpf-dependent population would not be revealed by EBA studies and constitutes a potentially important feature of the therapeutic response. Clearly it will be of paramount importance to determine whether this feature is related to the clinical response and subsequent relapse rates. Although the results from samples taken after the first week of therapy reinforce the points made previously, in three instances positive cultures were obtained from samples in which no colonial or liquid culture growth was obtained without Rpf supplementation. These results combined with the substantial increases in yield shown by the RI values indicate the potential value of Rpf supplementation for diagnostic culture. Not only should it be possible to recover viable bacilli later in chemotherapy and potentially facilitate identification of emerging resistance, but it seems likely that Rpf stimulation could enhance the sensitivity of culture, particularly for smear-negative pulmonary cases. In our service this could decrease or even eliminate culture-negative pulmonary cases, which currently stand at about 20 adult cases per annum (20% of adult pulmonary cases in 2008) and thereby facilitate appropriate chemotherapy and epidemiological control.

Although the degree to which sputum provides a representative sample of the population that must be eliminated by treatment will have a bearing on the points we raise regarding response to chemotherapy, there is surely little doubt that the phenotype of Mtb in sputum is significant for the onward transmission of this highly successful pathogen. The finding that the majority of expectorated cells are Rpf dependent for their subsequent growth therefore begs the question concerning whether Rpf is required to establish growth in a new host, and adds a further dimension to our previous suggestion that the nonreplicating phenotype in sputum may reflect adaptation to transmission (4). Further studies in this area hold out the possibility of specifically preventing transmission of tuberculosis, a feature largely absent from BCG and other vaccines in development.

We conclude that, by demonstrating the presence of Rpf-dependent cells in clinical samples, our findings provide direct evidence of the relevance of these intriguing proteins to human infection. They also provide further evidence of the presence of a large nonreplicating cell population in sputum and the complex nature of the physiological adaptation of mycobacterial cells to infection *in vivo* and transmission to a new host. The

existence of this apparently dominant but standard culture-invisible Mtb population potentially transmitted from patients with tuberculosis should be widely recognized and offers important opportunities for the development of enhanced diagnostic methods and new insights into the *in vivo* biology of this global pathogen.

Conflict of Interest Statement: G.V.M. received \$10,001–\$50,000 from the East Midland Development Agency in industry-sponsored grants for the improvement of TB diagnostics, and holds a patent from the University of Aberystwyth for bacterial pheromones and uses thereof. O.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.R.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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