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An *ex vivo* culture model for screening drug activity against *in vivo* phenotypes of *Mycobacterium tuberculosis*

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Since the activity of drugs against *Mycobacterium tuberculosis* grown in microbiological culture can differ from their activity against bacteria present in infected tissues, compounds with optimal activity against *in vivo* phenotypes may be overlooked in drug-discovery programmes that rely on *in vitro* screens. The authors have investigated the use of an *ex vivo* cell-culture model to assess the action of drugs on *M. tuberculosis* in an environment resembling that encountered during infection. Mycobacterial viability in the *ex vivo* model was shown to be regulated by the cell-mediated immune system, with growth inhibited by $CD4^+$ T cells at an early stage of infection in BCG-vaccinated mice, and at a later stage after infection in naive mice. Screening of drugs in the *ex vivo* model demonstrated a window of pyrazinamide susceptibility that coincides with the onset of the T-cell-mediated immune response in naive or vaccinated mice. It is proposed that pyrazinamide acts on a population of bacteria that are exposed to an acidic environment as a result of immune activation. Clinically, administration of pyrazinamide during the initial phase of treatment reduces the risk of relapse after 6 months, suggesting that the early pyrazinamide-susceptible population may contribute to the later pool of mycobacteria that persist during prolonged chemotherapy.

Keywords: tuberculosis, drug screening, pyrazinamide, luciferase

INTRODUCTION

With an estimated annual mortality of several million and a growing incidence of drug-resistant disease, there is a pressing need for new drugs to assist in the global control of tuberculosis (Dye *et al.*, 1999; Espinal *et al.*, 2001). At the same time, the development of molecular genetic tools for studying slow-growing mycobacterial pathogens, together with genome sequence data and modern combinatorial chemistry, provides unprecedented opportunities for drug-discovery programmes (Cole *et al.*, 1998; Barry *et al.*, 2000; Glickman & Jacobs, 2001).

In the conventional approach to drug discovery, compounds are initially optimized for activity against mycobacteria growing in *in vitro* culture and subsequently assessed for *in vivo* activity in animal models during preclinical development (Global Alliance for TB Drug Development, 2001). A limitation of this approach is that compounds that are particularly active against *in vivo* phenotypes of mycobacteria may be overlooked in the early discovery phase. The anatomical location and physiological status of mycobacteria within the infected host remains a topic of active research (McKinney, 2000). The requirement for prolonged chemotherapy to prevent relapse suggests that, in addition to the population of actively dividing mycobacteria present in the lungs of tuberculosis patients, there is a subset of non-dividing organisms that remain relatively resistant to the action of the drugs. This persistent population may include intracellular bacteria held under control by the immune response, as well as oxygen-deprived bacteria sequestered within fibrotic caseous lesions. The aim of the present study was to develop a cell-culture model in

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Abbreviations: BCG, bacille Calmette–Guérin; FCS, fetal calf serum; IFNγ, interferon-γ; r.l.u., relative light units.

which we could reproduce aspects of the complex environment encountered by *Mycobacterium tuberculosis* during infection, and which could be used for early assessment of the *in vivo* activity of novel drugs. As a starting point, we wished to establish a culture system that reflected the influence of host immunity on mycobacterial viability.

The effect of the host immune response on mycobacterial growth has been extensively characterized in intact animal models (Orme & Collins, 1994). During the initial acute stage of infection in naive mice, mycobacterial numbers increase over a period of several weeks prior to the induction of a mature cell-mediated immune response. This results in a reduction or cessation of bacterial multiplication, leading to a chronic phase of the disease during which bacterial load and mortality depend on the genetic background of the animal and on the initial dose and route of infection. Prior vaccination with BCG – a live attenuated variant of *Mycobacterium bovis* – results in accelerated induction of the immune response, reducing the extent of bacterial multiplication in the acute phase, and prolonging survival.

In the first part of this study we have demonstrated that mycobacterial viability in cell cultures prepared from tissues of infected mice is determined by the activity of the cell-mediated immune response. In the second part, we have analysed the effect of this immune-mediated control on the antimycobacterial activity of current drugs, and demonstrated a synergy between pyrazinamide and an active immune response.

METHODS

Mycobacterial strains and culture. *Mycobacterium tuberculosis* H37Rv and *Mycobacterium bovis* BCG, both carrying the pSMT1 luciferase reporter construct, were prepared and cultured as described previously (Snewin *et al*., 1999). Colony forming units (c.f.u.) were determined by plating on Middlebrook 7H11 plates supplemented with oleic acid, albumin, glucose and catalase, with 50 μg hygromycin ml^{-1} and 10 μg amphotericin B ml^{-1}. Plates were incubated at 37 °C in bags to prevent drying. Luminescence was measured in a class I biosafety cabinet using a Labsystems TL-Plus luminometer. The substrate, 0·1 ml 1 % (v/v) n-decyl aldehyde (Sigma) in ethanol, was injected into diluted cultures (1 : 1 with PBS) with a final volume of 1 ml, and the results expressed as relative light units (r.l.u.). To measure the effect of drugs on *in vitro* cultures of *M. tuberculosis*, exponential-phase cultures (OD_{600} 0·2) were inoculated into 25 ml RPMI culture medium [RPMI 1640 medium (Gibco-BRL) supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS, LabTech), 5 mM L-glutamine, 10 mM HEPES, 50 μM 2-mercaptoethanol, 50 U penicillin G ml^{-1} and 10 μg amphotericin B ml^{-1}]. Rifampicin, isoniazid or pyrazinamide (all from Sigma) were added to the cultures which were grown for 6 days in NUNC 24-well plates without shaking at 37 °C with 5 % (v/v) CO_2, and triplicate wells assayed for r.l.u.

Infection model and *ex vivo* culture. Female C57BL/6 mice (6–8 weeks old) were infected with *M. tuberculosis*/pSMT1 (5×10^5 c.f.u.) or BCG Pasteur/pSMT1 (5×10^6 c.f.u.) by intravenous injection in the tail. In experiments to monitor the effect of vaccination, groups of mice were vaccinated by subcutaneous injection of 5×10^5 c.f.u. BCG or saline, and challenged by intravenous injection 8–10 weeks later. Groups of 3–5 mice were killed by cervical dislocation, and spleens were removed aseptically and disaggregated by passing through a 100 mm cell strainer (Becton Dickinson, 2360), to produce a single-cell suspension in RPMI culture medium. Samples from individual spleens were serially diluted in PBS and plated out for c.f.u. and r.l.u. measured in triplicate. Splenocyte suspensions from individual mice generally gave luminescence readings differing by less than 20 %. Splenocytes with very low luminescence were occasionally found. These were assumed to result from failure of intravenous inoculation and were discarded. The remaining samples were pooled. Red blood cells were lysed using 0·83 % (w/v) ammonium chloride (pH 7·6), and splenocytes washed twice with PBS containing 10 % (v/v) FCS and once with RPMI culture medium. A sample was stained using 0·4 % (w/v) Trypan Blue solution and counted in an Improved Neubauer haemocytometer, from which total cell counts per spleen were calculated. Splenocyte preparations were then resuspended in culture medium at 1×10^6 cells ml^{-1}, seeded in NUNC 24-well tissue-culture dishes, and incubated at 37 °C with 5 % (v/v) CO_2 for 2–8 days. Analysis of the cell composition of splenocyte preparations revealed a predominance of B lymphocytes (50–60 % of total cells), with 14–15 % $CD4^+$ T cells and 11–12 % $CD8^+$ T cells.

Stocks of rifampicin (1 mg ml^{-1}), isoniazid (10 mg ml^{-1}) and pyrazinamide (10 mg ml^{-1}) were prepared in RPMI culture medium and added to splenocyte preparations before distribution into 24-well tissue culture dishes so that the final concentrations were 1, 0·1 and 0·01 μg ml^{-1} for rifampicin and isoniazid, and 500, 50 and 5 μg ml^{-1} for pyrazinamide. In some experiments splenocytes from uninfected mice were prepared in the same way and infected *in vitro* with BCG (1×10^4 c.f.u. ml^{-1}) either at the start of the incubation period or after 4 days in culture.

To monitor mycobacterial viability in *ex vivo* cultures, cells were lysed using 0·1 % (v/v) Triton X-100, r.l.u. measured and c.f.u. plated out from triplicate wells. Cultures were also examined by light microscopy after staining by the Kinyoun (cold) acid-fast procedure.

Immune manipulation: T cell depletion. T cell subsets were selectively depleted from *ex vivo* splenocyte cultures by complement-mediated cell lysis. Splenocyte samples were resuspended at 1×10^7 cells ml^{-1} and left for 1 h at 4 °C with a 1/100 dilution of anti-$CD4^+$ and/or anti-$CD8^+$ antibodies (monoclonal antibodies RL172.4 and 31M were kindly provided by Dr Ingrid Muller, Department of Immunology, Imperial College of Science, Technology and Medicine). After washing, rabbit complement (Cedarlane) was added at 1/10 dilution and incubated for 30 min at 37 °C. After two washes with PBS/FCS (10 %, v/v) and one wash with RPMI culture medium, cells were resuspended in RPMI culture medium at 1×10^6 ml^{-1}, and seeded in 24-well dishes as for the *ex vivo* method. Controls where no antibody was added and controls with no complement added were included in each experiment. A sample of each preparation was retained for flow cytometry analysis; these samples were stained for $CD4^+$ and $CD8^+$ T cell populations (antibodies from Sigma), fixed and analysed using a FACSCalibur Flow Cytometer (Becton Dickinson). Complement-mediated cell lysis typically achieved between 60 and 80 % depletion in the total cell number for the targeted T cell subset.

Interferon-γ assay. Interferon-γ (IFNγ) was measured in

supernatants removed from *ex vivo* cultures by ELISA (Pharmingen OptEIA kit).

Statistical analysis. Error bars in figures, and error terms for values reported in tables, show one standard deviation from the mean assessed in at least triplicate samples and experiments were repeated at least once. *P* values for Table 2 were calculated using a two-tailed paired Student's *t*-test.

RESULTS

Growth of luminescent mycobacteria

M. bovis BCG expressing a luciferase reporter gene was used in initial experiments to identify appropriate culture conditions. Luminescence output is dependent on bacterial viability and provides a rapid readout that reflects the number of c.f.u. (Snewin *et al.*, 1999). Intravenous challenge with BCG follows an analogous course to *M. tuberculosis*, with the initial phase of bacterial growth in the spleen of naive mice being controlled by the primed immune response in vaccinated animals. In initial experiments, cell cultures prepared from spleens removed from naive or vaccinated mice were infected with BCG *in vitro*. Infection of freshly prepared splenocytes resulted in unrestricted bacterial growth irrespective of prior vaccination status, as illustrated by the progressive increase in luminescence during a 1 week incubation period (Fig. 1a). Reduced bacterial growth was observed if splenocytes were maintained in tissue culture conditions for 4 days prior to infection, but under these conditions no difference was observed between naive and vaccinated splenocytes (data not shown). In contrast, the pattern of differential control of the infection seen in intact animals was reproduced in splenocyte cultures prepared from mice that had been infected with the luminescent strain *in vivo* (Fig. 1b). The same pattern was observed when measurement of c.f.u. was used as readout in place of the rapid assessment of luminescence. Control of mycobacterial growth in the vaccinated splenocytes indicated an influence of the acquired, T-cell-mediated immune response. Assay of IFNγ in supernatants from *ex vivo* splenocyte cultures produced results consistent with this hypothesis, with significant levels of IFNγ present in splenocyte cultures from vaccinated mice but not from naive or uninfected animals (Fig. 1c).

Visual inspection revealed formation of multicellular structures during the *ex vivo* culture period. Acid-fast staining showed that mycobacteria were predominantly associated with adherent splenocytes, with markedly higher numbers in naive as compared to vaccinated splenocytes.

The pattern of differential growth in naive and vaccinated splenocytes was dependent on the time at which the mice were killed (Fig. 2). Partial inhibition of mycobacterial growth could be detected in spleens removed from vaccinated mice as early as 1 day after infection, with a stronger inhibition evident by day 7. Two weeks after infection, mycobacterial growth was controlled in both naive and vaccinated splenocytes, although the total bacterial load was higher in the naive cultures. The timecourse for development of mycobacterial growth inhibition matched that of total cell recruitment measured in spleens from the naive and vaccinated mice (Fig. 2d).

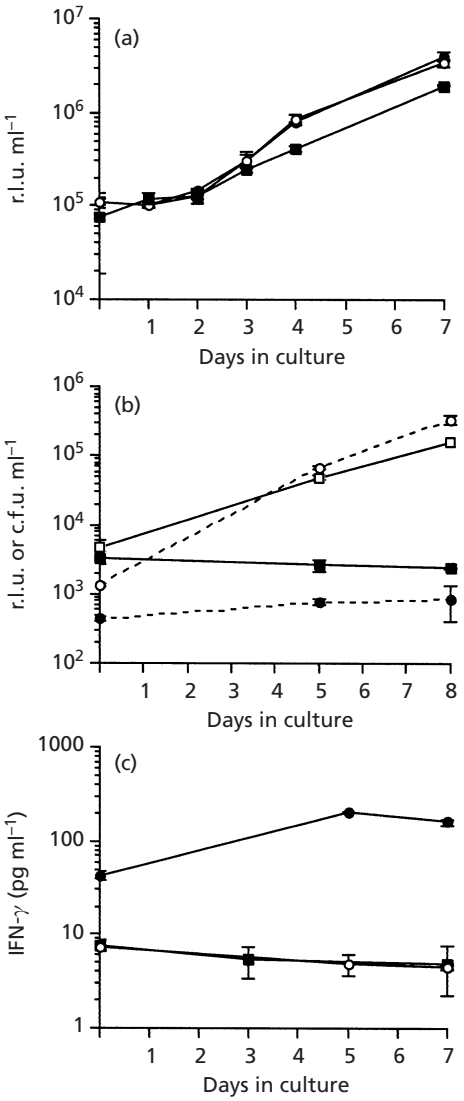

Fig. 1. Establishment of the *ex vivo* culture model. Single-cell splenocyte cultures were seeded in tissue culture wells at a density of 1×10^6 cells ml^{-1}. (a) *In vitro*. Splenocytes were infected with BCG/pSMT1 (1×10^4 c.f.u. ml^{-1}) on the first day of culture and mycobacterial viability, expressed as luminescent activity (r.l.u. ml^{-1}), was monitored over a 1 week incubation. Results are shown for splenocytes from naive mice (○) and from mice that were vaccinated with BCG 8–10 weeks previously (●). Growth in culture medium without cells is also shown (■). (b) *Ex vivo*. Splenocyte cultures were prepared from mice that had been infected with 1×10^6 c.f.u. BCG/pSMT1 3 days prior to being killed, and mycobacterial viability was monitored over a 1 week incubation. Results in splenocytes from naive mice (○, □) and vaccinated mice (●, ■) are expressed as r.l.u. (broken line) and c.f.u. (solid line) readouts. (c) IFNγ was measured in supernatants from *ex vivo* splenocyte cultures from vaccinated (●) and naive (○) mice, and from uninfected naive controls (■).

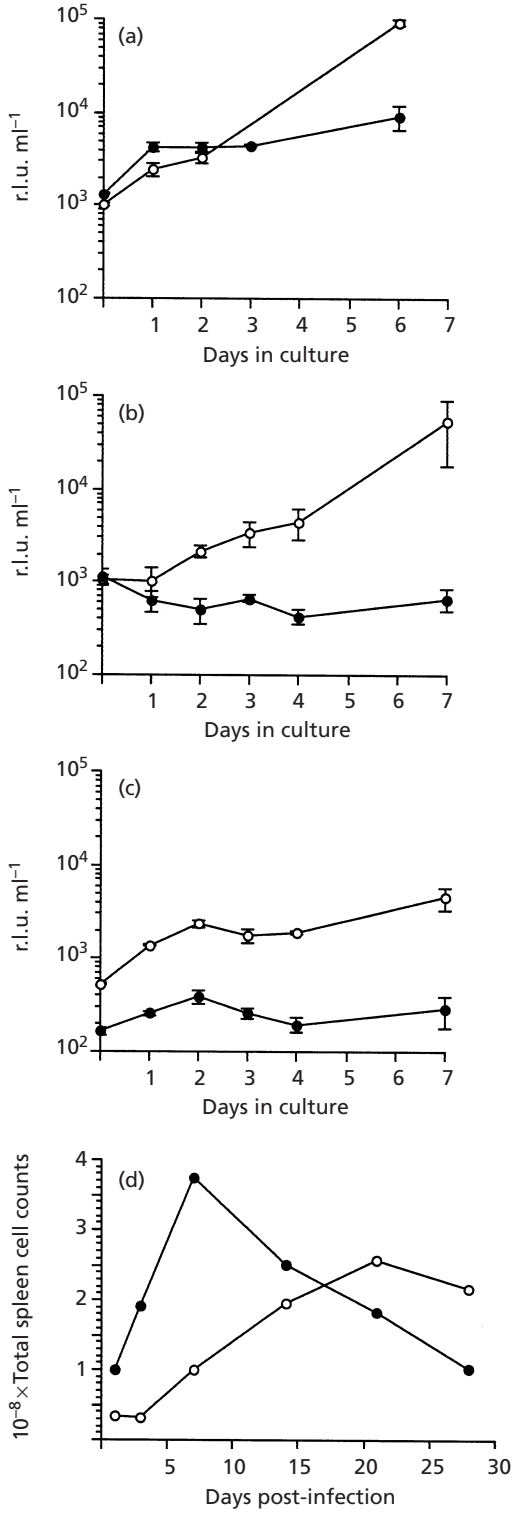

Fig. 2. Control of mycobacterial growth at different times after infection. Mice were killed 1 day (a), 7 days (b) or 14 days (c) after infection with BCG/pSMT1 and *ex vivo* cultures set up as described for Fig. 1. Control of mycobacterial growth was seen at early timepoints in splenocytes from vaccinated mice (●), but only after 2 weeks in naive splenocytes (○). Panel (d) shows the total cell counts in spleens from the two groups of mice; the timecourse of cell recruitment coincides with the ability to control mycobacterial growth.

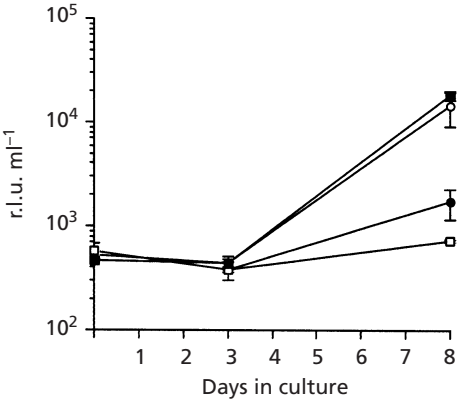

Fig. 3. Effect of T cell depletion in the *ex vivo* culture model. Splenocytes prepared from BCG-vaccinated mice infected 7 days previously with BCG/pSMT1 were subjected to T cell depletion as described in Methods. Control of mycobacterial growth in undepleted cultures (●) was lost after removal of $CD4^+$ T cells (○). Depletion of $CD8^+$ T cells (□) was associated with a slight reduction in mycobacterial growth. Growth in cultures depleted for both T cell subsets (■) was identical to that observed following $CD4^+$ depletion. IFNγ levels in the splenocyte cultures at the end of the 7 day incubation period were (pg ml^{-1}): undepleted, 105 ± 21; $CD4^+$-depleted, 20 ± 3; $CD8^+$-depleted; 102 ± 14; $CD4^+/CD8^+$-depleted, 20 ± 3.

Table 1. Comparison of *M. tuberculosis* and BCG in the *ex vivo* model

Splenocyte cultures were established from mice killed at different times after infection with luminescent BCG or *M. tuberculosis*. Luminescence was measured over 7 days of *ex vivo* culture and recorded as fold change in r.l.u. (i.e. r.l.u. reading at day 7 divided by r.l.u. reading at the start of the culture).

Days post infection	Fold change in r.l.u	
	Naive	Immunized
BCG infection		
1	93·1 ± 6·8	6·9 ± 1·9
7	31·2 ± 7·2	< 1 ± 0·5
14	8·1 ± 1·7	1·7 ± 0·7
***M. tuberculosis* infection**		
4	283·0 ± 24·5	85·9 ± 48·3
7	244·2 ± 33·8	9·3 ± 3·1
13	7·6 ± 1·7	7·3 ± 4·7

Contribution of T cell subsets

To determine the contribution of different T cell subsets to the control of mycobacterial growth, complement-mediated lysis was used to deplete $CD4^+$ or $CD8^+$ T cells from infected splenocytes prior to the *in vitro* culture step. T cell depletion had no effect on mycobacterial growth in the naive splenocytes prepared 7 days post-

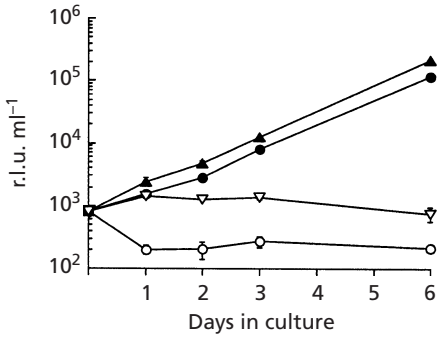

Fig. 4. Effect of drugs on luminescence of *M. tuberculosis*/pSMT1 *in vitro*. *M. tuberculosis*/pSMT1 cultures were set up in growth medium (▲), with the addition of isoniazid (1 μg ml^{-1}; ○), pyrazinamide (500 μg ml^{-1}; ●) or rifampicin (1 μg ml^{-1}; ▽). The effect of the drugs on mycobacterial viability was assessed by luminescence measurement.

infection. In the corresponding vaccinated splenocytes, however, depletion of CD4$^+$ T cells resulted in loss of the ability to control mycobacterial growth (Fig. 3). Depletion of CD8$^+$ T cells had no statistically significant effect, although a trend towards reduced mycobacterial growth was observed in some experiments. We cannot discount the possibility that the population of CD8$^+$ T cells remaining after depletion makes a contribution to control of bacterial growth. Treatment with complement alone, or with antibody in the absence of complement, had no effect on mycobacterial growth. Assay of IFNγ in supernatants from these *ex vivo* splenocyte cultures showed that levels were reduced following CD4$^+$ T cell depletion (see Fig. 3 legend).

BCG versus *M. tuberculosis*

Splenocyte cultures from mice infected with luciferase-expressing *M. tuberculosis* showed a pattern similar to that observed with BCG, with early containment of bacterial growth in splenocytes prepared from vaccinated animals (Table 1). While *ex vivo* cultures infected with BCG could be maintained over 1 week, the corresponding *M. tuberculosis* cultures showed signs of splenocyte cell deterioration at late timepoints, and subsequent experiments with *M. tuberculosis* focused on bacterial viability during the initial 2 day stage of *ex vivo* culture.

Effect of drugs in the *ex vivo* model

Effect of drugs on luminescence. The effect of drugs on luminescent output of the reporter strain was tested by adding isoniazid, rifampicin or pyrazinamide to *M. tuberculosis*/pSMT1 cultures in the RPMI culture me-

Table 2. Summary of the effect of drugs in the *ex vivo* assay

The effect of adding different concentrations of each of three antimycobacterial drugs [isoniazid (INH), pyrazinamide (PZA) and rifampicin (Rif)] is shown for splenocyte cultures prepared at three different timepoints after infection of mice with *M. tuberculosis*/pSMT1. The results are expressed as the percentage reduction in mycobacterial viability in drug-treated as compared to control cultures after 2 days in the *ex vivo* assay. Viability was assessed by luminescence (r.l.u.) or by subsequent growth in microbial culture (c.f.u.). Addition of pyrazinamide at 50 μg ml^{-1} resulted in a significant inhibition of growth of *M. tuberculosis* in 21-day cultures as measured by r.l.u. ($P<0{\cdot}01$) or by c.f.u. ($P<0{\cdot}04$); there was no significant inhibition at the 7-day and 42-day timepoints. At 500 μg ml^{-1}, pyrazinamide caused some inhibition at each of the timepoints, but this was significantly greater (r.l.u. $P<0{\cdot}009$, c.f.u. $P<0{\cdot}03$) at 21 days than at 7 or 42 days. The r.l.u. readings in untreated cultures were: day 7, 24500 ± 1240; day 21, 4080 ± 69; day 42, 1970 ± 81. The c.f.u. readings in untreated cultures were: day 7, 8533 ± 635; day 21, 3260 ± 66; day 42, 2380 ± 74. ND, Not done.

Drug concn (μg ml^{-1})	7 days post infection		21 days post infection		42 days post infection	
	r.l.u	c.f.u	r.l.u	c.f.u	r.l.u	c.f.u
INH						
1	92·4 ± 0·9	98 ± 0·1	73 ± 1·7	68·8 ± 2·4	82·4 ± 1·5	90·6 ± 0·9
0·1	91·6 ± 0·4	93·3 ± 0·5	67·8 ± 1·2	58·2 ± 0·9	78·8 ± 3·2	73·4 ± 4·1
0·01	< 1	< 1	10·5 ± 0·5	8·1 ± 18·4	10·8 ± 5·6	< 1
PZA						
500	19·6 ± 4	30·5 ± 7·7	59·3 ± 1·1	68 ± 1·8	22·7 ± 4·5	33·4 ± 3·4
50	1·6 ± 2·4	3·1 ± 7·9	29·1 ± 1·4	52·2 ± 5·2	3 ± 6·7	12·4 ± 6·6
5	8·7 ± 3·6	19·5 ± 0·8	3·4 ± 2·8	28·6 ± 14·3	< 1	< 1
Rif						
1	64·5 ± 2·4	96·9 ± 0·8	44·1 ± 0·6	92·9 ± 0·9	49·3 ± 0·4	96·3 ± 6
0·1	16·7 ± 0·4	ND	16 ± 3·1	70·4 ± 1·1	2·7 ± 5·3	55·3 ± 3·4
0·01	5·3 ± 0·2	ND	3·3 ± 2·2	24·5 ± 11·4	< 1	2 ± 28·3

Table 3. Effect of vaccination on pyrazinamide activity

The effect of pyrazinamide in the *ex vivo* assay was tested using splenocytes from naive or vaccinated mice infected 11 days previously with *M. tuberculosis*/pSMT1. Results are shown as c.f.u. readout.

PZA (μg ml^{-1})	c.f.u. ml^{-1} after 2 days in culture	
	Naive	Vaccinated
0	$5{\cdot}8\ (\pm 0{\cdot}4) \times 10^3$	$8{\cdot}6\ (\pm 0{\cdot}6) \times 10^2$
50	$8{\cdot}4\ (\pm 0{\cdot}2) \times 10^3$	$4{\cdot}0\ (\pm 2{\cdot}0) \times 10^2$
500	$7{\cdot}5\ (\pm 0{\cdot}3) \times 10^3$	$2{\cdot}0\ (\pm 1{\cdot}0) \times 10^2$

dium (Fig. 4). Under these conditions, there was a sharp fall in r.l.u. following exposure to isoniazid, rifampicin inhibited the increase in r.l.u. seen in control cultures, and pyrazinamide had no effect on r.l.u. Over the same period, the number of c.f.u. from cultures treated with isoniazid or rifampicin fell by more than 90 % over 48 h, but pyrazinamide had no effect on c.f.u. The differential effects on c.f.u. and r.l.u. measurements reflect differences in the mode of action of the drugs, and led us to include both readouts in subsequent experiments.

Effect of drugs during different stages of infection. We next tested the effect of addition of each of the drugs in the *ex vivo* assay. Table 2 illustrates an experiment using splenocytes harvested at different timepoints after *M. tuberculosis* infection. The results are expressed as the percentage inhibition of r.l.u. and c.f.u. readings after 2 days of *ex vivo* culture in the presence or absence of drugs. Isoniazid and rifampicin strongly inhibited mycobacterial growth at each of the timepoints; although consistent with the *in vitro* observations described above, the effect of rifampicin was most obvious in terms of the c.f.u. readout. Pyrazinamide had no effect at day 7, but had a significant inhibitory effect at day 21. This effect was reduced by the 42 day timepoint. There was a distinct time window between 2 and 4 weeks after infection, during which pyrazinamide was effective. The inhibitory activity of isoniazid and rifampicin was slightly lower during this timeframe.

The timing of pyrazinamide activity in the *ex vivo* model coincided with the induction of a cell-mediated immune response. To further explore the possible association between these two events, the effect of pyrazinamide was compared in *ex vivo* splenocytes from naive and BCG-vaccinated mice 11 days after infection with *M. tuberculosis* (Table 3). This timepoint was chosen to represent the stage of the infection at which a cell-mediated immune response was evident in vaccinated but not naive animals (see Fig. 2). While pyrazinamide had no effect on mycobacterial viability in splenocytes from naive mice at this timepoint, a clear dose-dependent inhibition was seen in the cultures from vaccinated mice.

DISCUSSION

A range of assays are available to test the ability of drugs to inhibit the *in vitro* growth of mycobacteria, but assessment of their *in vivo* activity is dependent on costly and time-consuming experiments in intact animals (Global Alliance for TB Drug Development, 2001). We have explored the possibility of establishing a cell-culture model that would allow screening of activity against mycobacteria exposed to conditions resembling those encountered during infection. The viability of mycobacteria in *ex vivo* cultures prepared from spleens of infected mice was shown to be determined by the host immune response. Mycobacterial growth was unrestricted in the absence of acquired cell-mediated immunity in naive animals, but was inhibited by the immune response primed by prior BCG vaccination. Inhibition was relieved by removal of $CD4^+$ T cells, consistent with *in vivo* evidence of the key role for these cells in control of the initial acute phase of mycobacterial infection (Flynn & Chan, 2001), and was associated with secretion of IFNγ. At later stages of the infection, a similar control of mycobacterial growth was observed in splenocytes prepared from initially naive mice. The *ex vivo* model offers the potential for further analysis of immunological aspects of mycobacterial pathogenesis. It will be of interest to test the effect of depletion or addition of various cytokines or lymphocyte subsets at different stages of the infection, for example, and to use this model in parallel with experiments in whole animals to dissect the role of different immune mechanisms in the control of tuberculosis. An attractive aspect of the *ex vivo* model is that multiple experimental variables can be tested using only a limited number of animals; up to 100 individual cultures can be established from a single spleen.

We have exploited the *ex vivo* model to study the influence of host immunity on the action of antimycobacterial drugs. These experiments were monitored by assessment of c.f.u. in addition to the rapid luminescence readout used in the immunology experiments. Drugs differ in their effect on luminescence output in short-term assays. Isoniazid triggers a rapid drop in r.l.u., for example, but this is not the case for rifampicin. The reporter construct used in the assay constitutively expresses a high level of the luciferase enzyme and the drop in r.l.u. suggests that isoniazid induces rapid depletion of the reduced FMN cofactor required for luminescence (Meighen, 1991), possibly related to changes in the permeability of the mycobacterial cell wall. The continued luminescence of rifampicin-treated cultures indicates availability of a pool of cofactor within the mycobacteria which may persist for some days. Luminescence provides a measure of the amount of luciferase enzyme and the energy balance in the bacterium. This is not directly related to replication, although decreased luminescence may provide an early indication of the fact that replication is unlikely to take place. Therefore, although there is a consistent correlation between r.l.u. and c.f.u. in actively growing cultures (Snewin *et al.*, 1999), this is not necessarily

preserved when mycobacteria are being killed. It is important to note that luminescence provides an immediate measure of the physiological status of the bacteria, while the ability to form colonies is influenced by drug-induced changes that occur during the subsequent weeks in culture.

The most striking observation from the assay of drug activity in the *ex vivo* model was the change in the effect of pyrazinamide at different times after infection. Pyrazinamide had maximal activity in splenocyte cultures prepared at the time of onset of the antimycobacterial immune response. Pyrazinamide is an important component of current treatment regimens but is unique amongst tuberculosis drugs in being ineffective against bacteria growing in conventional laboratory culture. It does reduce the viability of *in vitro* cultures exposed to acidic pH (Heifets & Lindholm-Levy, 1992), and has some inhibitory effect when administered in the mouse model (Klemens *et al*., 1996), although there are conflicting reports of its ability to influence growth of intracellular mycobacteria in macrophage cell cultures (Crowle *et al*., 1986; Heifets *et al*., 2000). Our experimental observations are consistent with a model in which pyrazinamide acts against a population of mycobacteria exposed to an acidic environment as a result of the action of the host immune response (Mitchison, 1985). While interference with phagosome maturation is considered the predominant strategy for mycobacterial survival in macrophages (Russell, 2001), the pyrazinamide-susceptible population may represent some subset of bacteria that are present within acidified phagolysosomes. It is interesting to note that the window of pyrazinamide susceptibility does not extend into the chronic phase of infection in the mouse model. Although there is evidence of a continued cell-mediated immune response at 42 days, our observations indicate that this does not generate a pyrazinamide-susceptible population of bacteria. This suggests that acidic conditions do not provide the environment for persistence, although it does not exclude the possibility that bacteria that survive exposure to acidic conditions subsequently contribute to the persistent pool. This model is of interest in relation to the clinical use of pyrazinamide. Pyrazinamide is included during the initial 2 month phase of therapy and has been shown to reduce the risk of relapse at the end of the standard 6 month regimen (Global Alliance for TB Drug Development, 2001). This profile is consistent with an early effect of pyrazinamide on the development of persistent organisms rather than a direct activity against an established persistent population.

While the simple *ex vivo* model described in the present study has been useful in exploring the window of pyrazinamide susceptibility, there is considerable scope for future refinement. Preliminary experiments indicate that a similar approach can be applied to infected lung tissues, for example, and modification of cell-culture techniques may extend the length of the assay period by reducing the lytic effects observed in *M. tuberculosis* cultures. The limitations of the luciferase readout as a marker for drug activity suggest a potential role for alternative reporter constructs in further optimization of the *ex vivo* approach. An attractive strategy is to engineer expression of the reporter gene under the control of promoters induced in response to drug action (Wilson *et al*., 1999). This approach has been successfully employed in incorporating the *ex vivo* assay into a development programme for improved ethambutol derivatives (C. E. Barry, personal communication).

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REFERENCES

Barry, C. E., 3rd, Slayden, R. A., Sampson, A. E. & Lee, R. E. (2000). Use of genomics and combinatorial chemistry in the development of new antimycobacterial drugs. *Biochem Pharmacol* **59**, 221–231.

Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.

Crowle, A. J., Sbarbaro, J. A. & May, M. H. (1986). Inhibition by pyrazinamide of tubercle bacilli within cultured human macrophages. *Am Rev Respir Dis* **134**, 1052–1055.

Dye, C., Scheele, S., Dolin, P., Pathania, V. & Raviglione, M. C. (1999). Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* (*J Am Med Assoc*) **282**, 677–686.

Espinal, M. A., Laszlo, A., Simonson, L. & 9 other authors (2001). Global trends in resistance to antituberculosis drugs. World Health Organization–International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. *N Engl J Med* **344**, 1294–1303.

Flynn, J. L. & Chan, J. (2001). Immunology of tuberculosis. *Annu Rev Immunol* **19**, 93–129.

Glickman, M. S. & Jacobs, W. R., Jr (2001). Microbial pathogenesis of *Mycobacterium tuberculosis*: dawn of a discipline. *Cell* **104**, 477–485.

Global Alliance for TB Drug Development (2001). Scientific blueprint for tuberculosis drug development. *Tuberculosis* **81, Suppl 1**, 1–52.

Heifets, L. & Lindholm-Levy, P. (1992). Pyrazinamide sterilizing activity *in vitro* against semidormant *Mycobacterium tuberculosis* bacterial populations. *Am Rev Respir Dis* **145**, 1223–1225.

Heifets, L., Higgins, M. & Simon, B. (2000). Pyrazinamide is not active against *Mycobacterium tuberculosis* residing in cultured human monocyte-derived macrophages. *Int J Tuberc Lung Dis* **4**, 491–495.

Klemens, S. P., Sharpe, C. A. & Cynamon, M. H. (1996). Activity of pyrazinamide in a murine model against *Mycobacterium tuberculosis* isolates with various levels of *in vitro* susceptibility. *Antimicrob Agents Chemother* **40**, 14–16.

McKinney, J. D. (2000). *In vivo veritas*: the search for TB drug targets goes live. *Nat Med* **6**, 1330–1333.

Meighen, E. (1991). Molecular biology of bacterial luminescence. *Microbiol Rev* **55**, 123–142.

Mitchison, D. A. (1985). The action of antituberculosis drugs in short-course chemotherapy. *Tubercle* **66**, 219–225.

Orme, I. M. & Collins, F. M. (1994). Mouse model of tuberculosis. In *Tuberculosis*: *Pathogenesis*, *Protection and Control*, pp. 113–134. Edited by B. R. Bloom. Washington, DC: American Society for Microbiology.

Russell, D. G. (2001). *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol* **2**, 569–577.

Snewin, V. A., Gares, M. P., Gaora, P. O., Hasan, Z., Brown, I. N. & Young, D. B. (1999). Assessment of immunity to mycobacterial infection with luciferase reporter constructs. *Infect Immun* **67**, 4586–4593.

Wilson, M., DeRisi, J., Kristensen, H. H., Imboden, P., Rane, S., Brown, P. O. & Schoolnik, G. K. (1999). Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. *Proc Natl Acad Sci U S A* **96**, 12833–12838.

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