The immunogenicity of recombinant *Mycobacterium smegmatis* bearing BCG genes

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Specific pathogen-free C57BL/6 mice infected with recombinant *Mycobacterium smegmatis* (rM. *smegmatis*) bearing BCG genes showed increased splenic survival compared to those receiving the vector control (plasmid DNA only). The mouse-passaged rM. *smegmatis* (J3R) survived in peritoneal macrophages better than the vector control, regardless of whether the macrophages were infected *in vivo* or *in vitro*. When rM. *smegmatis* J3R was cultured in synthetic Proskauer-Beck-Tween medium, protein bands characteristic of BCG culture filtrates and not present in the vector control preparation were observed. Mice immunized with two doses of heat-killed J3R suspended in Freund's adjuvant were able to limit the growth of virulent *Mycobacterium tuberculosis* within the lung and spleen compared to that observed in control mice receiving adjuvanted vector control or Freund's adjuvant alone.

Keywords: *Mycobacterium smegmatis*, BCG genes, macrophages

INTRODUCTION

Bacille Calmette–Guérin (BCG) has been used for more than 70 years for the prevention of pulmonary tuberculosis, especially in children living in Third World countries. Despite the widespread use of the BCG vaccine, as much as one third of the world's population is infected with tubercle bacilli, with 8 million new cases and nearly 3 million deaths being reported each year (Sudre *et al.*, 1992). As a result, tuberculosis is still the most important life-threatening bacterial disease in the world today and has recently been declared to be a global health emergency by the World Health Organization (WHO, 1993). Neonatal immunization with live BCG vaccine constitutes the major component of tuberculosis control programmes in many parts of the world, despite questions raised by several recent field trials regarding its overall protective value (Fine, 1989). As a result of uncertainties regarding the protective ability of currently available BCG vaccines, a number of proposals have been made to increase the

immunogenicity of existing BCG vaccines by cloning more protective genes and transferring them into suitable carriers (Bloom, 1989; Curtiss *et al.*, 1989). The recent development of the integrating shuttle cosmid (Snapper *et al.*, 1988) makes it possible to transfer genetic material from one mycobacterial species to another and could lead to the development of new, improved vaccines for use against this important human disease (Collins, 1991).

The present study examines the ability of recombinant *Mycobacterium smegmatis* (rM. *smegmatis*) selected *in vivo* and bearing BCG genes to produce protein antigens capable of enhancing resistance to the growth of virulent tubercle bacilli within the lungs of aerogenically challenged mice.

METHODS

Animals. Specific pathogen-free C57BL/6 mice (6–8 weeks old) were obtained from the Trudeau Animal Breeding Facility, Saranac Lake, New York. They were maintained under barrier conditions and fed sterilized commercial mouse chow and acidified water *ad libitum* (Collins, 1972).

Organisms. *Mycobacterium bovis* BCG Pasteur (TMC 1011) and *Mycobacterium tuberculosis* H37Rv (TMC 102) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, New York. They were grown in Proskauer-Beck-Tween (PBT) liquid medium enriched with 10% oleic acid/albumin/dextrose/catalase additive, harvested after incubation at 37 °C for 10 d (mid-exponential growth phase) and dispensed into

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Abbreviation: rM. *smegmatis*, recombinant *Mycobacterium smegmatis*.

1 ml ampoules which were kept frozen at -70°C until required (Kim & Kubica, 1972). *M. smegmatis* strain mc²155 was derived from *M. smegmatis* ATCC 607 (Snapper *et al.*, 1990) and was cultured in PBT medium at 37°C for 3 d before being stored at -70°C . The viability of the recombinants was checked by thawing randomly selected ampoules, sonicating the organisms briefly to disperse any clumps and plating tenfold saline dilutions on Middlebrook 7H11 agar (Difco) enriched with $10\ \mu\text{g}$ kanamycin ml^{-1} (Snapper *et al.*, 1988). Plates were incubated at 37°C in sealed plastic bags and the colonies counted using a plate microscope.

Construction of *M. smegmatis*/BCG recombinant pools. A genomic library from *M. bovis* BCG Pasteur, which had been grown as a surface pellicle on Proskauer and Beck medium for 14 d at 37°C , was prepared by mechanical disruption and phenol/chloroform extraction (Grosskinsky *et al.*, 1989). The DNA was digested into 30–50 kb fragments with *Sau*3A before being ligated to the integrating shuttle cosmid vector pYUB178 (Jacobs *et al.*, 1991). The pYUB178::BCG DNA or pYUB178 DNA was electroporated into *M. smegmatis* mc²155 which had been grown in PBT medium for 3 d at 37°C (Snapper *et al.*, 1988). Separate pools of approximately 250 transformants were selected from kanamycin agar (theoretically sufficient to represent the entire BCG genome) and picked into 50 ml Middlebrook 7H9 broth enriched with $10\ \mu\text{g}$ kanamycin ml^{-1} and incubated at 37°C for 3 d. The vector control (pYUB178) was prepared as a pool of 15 transformants. The recombinant pools were dispensed in 5 ml aliquots and stored at -70°C until needed.

Infection and enumeration of recombinant bacteria *in vivo*. A frozen ampoule was thawed, sonicated briefly to break up any clumps and suitable saline dilutions were injected intravenously into C57BL/6 mice. Each mouse received approximately 10^6 c.f.u. of each recombinant suspended in 0.2 ml saline. At intervals up to 14 d, groups of five randomly selected mice were killed by cervical dislocation, and selected organs were removed aseptically and homogenized separately in cold sterile saline before being plated on Middlebrook 7H11 agar enriched with $10\ \mu\text{g}$ kanamycin ml^{-1} . Colonies were counted after 3 d incubation at 37°C , representative rough, opaque colonies were checked for purity and a 3 d broth culture was stored at -70°C until required.

Challenge protocol. Groups of vaccinated and control mice were infected aerogenically with approximately 10^8 c.f.u. *M. tuberculosis* H37Rv using a Middlebrook chamber (Collins, 1985). The size of the challenge was checked by sacrificing five control mice 1 h later and plating the lung homogenate on Middlebrook 7H11 agar. The plates were incubated at 37°C in sealed plastic bags for up to 21 d and the average number of c.f.u. per organ was calculated. The standard counting error for five replicate determinations was usually less than 10% of the mean.

Infection of mouse macrophages *in vivo*. Peritoneal exudate macrophages were infected with mouse-passaged *M. smegmatis* J3R or vector control suspensions. The exudate was induced by the intraperitoneal injection of 2 ml 10% (w/v) casein in saline and the peritoneal cavity was washed out 3 d later using 5 ml RPMI 1640 medium enriched with 10% (v/v) heat-inactivated foetal calf serum (Gibco) plus 5 U heparin ml^{-1} (Stokes & Collins, 1988). The peritoneal exudate cell suspension was washed twice with RPMI plus 1% FCS and the number of viable cells was determined by trypan blue exclusion. The cells were suspended at a concentration of 2×10^6 cells per ml of RPMI 1640 medium containing 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 10 mM HEPES (complete

medium), dispensed into 24-well plates (Costar) and incubated at 37°C in 5% CO_2 -enriched humidified air for 24 h. Non-adherent cells were removed by gentle washing and at least 95% of the adherent cells were determined to be macrophages based on non-specific esterase staining (Hubbard & Collins, 1991).

Macrophage monolayers were infected with 10^8 c.f.u. mycobacteria suspended in 1 ml complete medium and incubated in CO_2 -enriched air for 4 h at 37°C . The monolayers were washed gently with RPMI plus 10% FCS before adding 1 ml complete medium containing $5\ \mu\text{g}$ gentamicin ml^{-1} (the MIC for *M. smegmatis*) and reincubating at 37°C for up to 48 h. At approximately 6 h intervals, coverslips were removed from three of the wells, washed twice with warm PBS (0.145 M NaCl, 0.15 M sodium phosphate, pH 7.4), fixed and then stained by the Ziehl–Neelsen method to determine the percentage of infected macrophages and their multiplicity of infection. Other coverslips were treated with naphthol blue black (Sigma) at a concentration of $0.5\ \text{mg}$ (ml 0.1 M citric acid)⁻¹ and 1% Triton X-100 (Stokes *et al.*, 1986). This solution lysed the adherent cells releasing any intracellular mycobacteria and the phagocyte nuclei. The bacteria were counted by immediately diluting 0.1 ml of the lysate in 0.9 ml 0.2 M phosphate buffer (pH 7.4), which was briefly sonicated to disperse any clumped bacteria, and plating on agar enriched with $10\ \mu\text{g}$ kanamycin ml^{-1} . Colonies were counted after 3 d incubation at 37°C . The number of macrophages per well was determined by counting the stained nuclei using a haemocytometer. Viable mycobacteria were expressed per 10^6 macrophages present on the monolayer at the time of harvest (Stokes *et al.*, 1986).

Infection of mouse macrophages *in vivo*. Normal mice were injected intraperitoneally with casein 3 d prior to inoculation with 10^6 c.f.u. *M. smegmatis* suspended in 0.5 ml 0.05% Tween/saline. The mice were sacrificed 10 min later and their peritoneal cavities washed out with 5 ml RPMI 1640 medium containing 10% FCS plus heparin. The cell suspension was washed, its viability was checked by trypan blue exclusion and diluted to 2×10^6 cells (ml complete medium)⁻¹. The infected cells were cultured in 24-well plates in 5% CO_2 -enriched air for 2 h when the monolayers were washed gently to remove non-attached cells before being replaced with complete medium containing $5\ \mu\text{g}$ gentamicin ml^{-1} . The average number of viable mycobacteria was calculated per 10^6 macrophages from three wells per time point.

***M. smegmatis* culture filtrate antigens.** The mycobacteria were grown in Proskauer–Beck–Tween medium without added albumin (Collins *et al.*, 1988) and harvested during the mid-exponential growth phase (3 d for *M. smegmatis* and 8–10 d for *M. bovis* BCG). Cells were removed by centrifugation at 10000 g for 30 min at 4°C and the supernatant fluid was sterilized by filtration through a 0.22 μm pore-size membrane (Millipore). Proteins were precipitated by 80% ammonium sulfate saturation at 4°C overnight. The precipitate was dissolved in 0.5 M PBS (pH 7.4) and dialysed in the cold until free of ammonium ions. The protein content was determined using the bicinchoninic acid assay (Pierce) against a bovine serum albumin standard (Hubbard *et al.*, 1992). Culture filtrate proteins were separated by SDS-PAGE run under reducing conditions in an 11% (w/v) acrylamide gel and stained with Coomassie blue (Collins *et al.*, 1988).

Statistical analysis. The viability data were analysed by Student's *t*-test or an analysis of variance for differences between two or several independent means using a Statview 512+ program (Brainpower, Calabases) run on a Macintosh SE computer.

RESULTS

Mouse passage of *M. smegmatis* (pYUB178::BCG) recombinants

C57BL/6 mice were infected intravenously with the *M. smegmatis* recombinant pool. Viable counts were carried out on spleen (Fig. 1) and lung homogenates (data not shown) at intervals up to 14 d. The number of viable recombinants present in the spleen declined steadily with time and did not resemble the growth of BCG in normal mice (Collins, 1984). Colonies were selected from the day 14 splenic homogenates plated on kanamycin agar, checked for purity and grown in 7H9 broth for 3 d, then stored at -70°C until required.

Growth of *rM. smegmatis* J3R in mouse macrophages

Peritoneal exudates were induced in C57BL/6 mice and the macrophage monolayers were infected 24 h later with approximately 10^6 c.f.u. mouse-passaged *rM. smegmatis* J3R or the vector control. The number of viable organisms was then determined at intervals up to 48 h. Both suspensions were rapidly taken up by the macrophages and few non-cell-associated organisms were detected in the stained monolayers (Fig. 2). The viability of the recombinant challenge declined steadily with time although usually at a slower rate than the vector control (Fig. 3). Little difference was seen in the percent infected macrophages in these monolayers or in the number of acid-fast bacilli (multiplicity of infection) per macrophage, even after 48 h incubation (Table 1). There was no evidence of intracellular growth by the recombinant in these macrophage monolayers, regardless of whether infection occurred *in vitro* (Fig. 3) or *in vivo* (Fig. 4).

Protein antigens present in *rM. smegmatis* J3R culture filtrates

When culture filtrates from *rM. smegmatis* J3R grown in PBT medium were examined for protein bands by SDS-PAGE, an intensely staining 65 kDa band was observed

Fig. 1. Viability of the *rM. smegmatis* pool (■) or vector control (●) in the spleens of C57BL/6 mice following intravenous inoculation with approximately 10^6 c.f.u. of each strain in 0.2 ml Tween/saline. The vertical bars represent the SEM for five determinations.

(Fig. 5, lane 3). Culture filtrates prepared from BCG were also characterized by a strong 65 kDa protein band (lane 2) which was virtually absent from the vector control preparation (lane 4). A number of higher molecular mass (80–120 kDa) bands were also present in the *rM. smegmatis* and BCG culture filtrates, which were not present in the corresponding vector control preparation. This suggests that the recombinant was able to express transferred BCG gene(s) and so a study of the protective ability of this organism was undertaken in appropriately vaccinated mice.

Protection of mice vaccinated with *rM. smegmatis* J3R

C57BL/6 mice were infected intravenously with approximately 10^8 c.f.u. *rM. smegmatis* J3R suspended in 0.2 ml saline. Three weeks later they were challenged aerogenically with 10^3 c.f.u. *M. tuberculosis* H37Rv, but the resulting growth curve indicated no increase in anti-

Table 1. Survival of *rM. smegmatis* J3R in mouse peritoneal macrophages

Time (h)	Strain*	Macrophage infection		Viable count (c.f.u. $\pm$ SD)	Survival (%)
		Percentage infected	Multiplicity		
0	J3R	65	1–5	$2.7 \pm 0.38 \times 10^4$	–
	VC	58	5	$1.6 \pm 0.87 \times 10^4$	–
6	J3R	ND	ND	$2.8 \pm 0.76 \times 10^4$	100
	VC	ND	ND	$1.2 \pm 0.20 \times 10^4$	75
24	J3R	ND	ND	$1.4 \pm 0.13 \times 10^4$	52
	VC	ND	ND	$5.3 \pm 2.10 \times 10^3$	33
48	J3R	50	5–10	$7.8 \pm 2.40 \times 10^3$	29
	VC	64	2–5	$1.5 \pm 0.25 \times 10^3$	10

ND, Not determined.

* J3R, *rM. smegmatis* J3R; VC, vector control.

Fig. 2. Peritoneal mouse macrophages infected with *rM. smegmatis* J3R after 24 h incubation at 37 °C. The monolayer was stained by the Ziehl-Neelsen method ($\times 1000$).

tuberculous resistance, even when vaccination was given as 3 weekly doses of live organisms prior to challenge (V. Falcone, unpublished data).

In a second vaccination protocol, the C57BL/6 mice were injected subcutaneously with two doses of 20 μ g (approximately 10^8 acid fast bacilli) heat-killed *rM. smegmatis* J3R suspended in 0.2 ml Freund's incomplete adjuvant given after a 3 week interval (Collins & Mackaness, 1970b). A group of control mice were vaccinated with a standard inoculum of live BCG Pasteur. The challenge inoculum in these mice was distinguished from the residual BCG by plating the lung homogenates on Middlebrook 7H11 agar containing 2 μ g thiophene-carboxylic acid hydrazide ml^{-1} (Collins & Mackaness, 1970a). Other controls received Freund's incomplete adjuvant alone or 20 μ g killed vector control suspended in Freund's incomplete adjuvant. The vaccinated mice

(together with age-matched unvaccinated controls) were challenged aerogenically with 10^8 c.f.u. *M. tuberculosis* 3 weeks later and the growth of these organisms in the lungs and spleen was compared with the controls (Fig. 6). Mice receiving two doses of adjuvanted *rM. smegmatis* showed a significant reduction in the number of viable *M. tuberculosis* present in the lungs ($P < 0.01$) and the spleen ($P = 0.05$) compared to the controls 28 d later. The reduction was only slightly less than that observed in the BCG vaccinated controls (Fig. 6).

DISCUSSION

Recent advances in gene cloning technology make it possible to transfer BCG genes to a variety of vectors, which will overproduce the specific protective antigen(s) to provide a long-lasting resistance to a subsequent tuberculous infection (Bloom, 1989). However, to be

Fig. 3. Survival of *M. smegmatis* J3R (Δ , $\blacktriangle$) and the vector control ($\square$, $\blacksquare$) in peritoneal macrophages infected *in vitro*. The vertical bars represent the SEM for three determinations.

fully effective, the vector must be capable of establishing a persistent, self-limiting infection within the reticulo-endothelial organs of the vaccinated host (Collins, 1991). Thus, the carrier must be able to survive *in vivo* for a long period of time without inducing detectable levels of immunopathology; the more persistent the infection, the better the protection (Curtiss *et al.*, 1989). The r*M. smegmatis* was eliminated from the mouse tissues at a slightly, though consistently slower rate than the vector

Fig. 4. Survival of r*M. smegmatis* J3R (Δ , $\blacktriangle$) and the vector control ($\square$, $\blacksquare$) in peritoneal macrophages infected *in vivo*. The vertical bars represent the SEM for three determinations.

Fig. 5. SDS-PAGE gel showing culture filtrate proteins obtained from BCG (lane 2), r*M. smegmatis* J3R (lane 3) or the vector control (lane 4) when stained with Coomassie blue. Protein standards are shown in lane 1.

control (Fig. 1). Different commercially available BCG vaccines vary extensively in their persistence (and immunogenicity) when tested in mice or guinea pigs under standardized laboratory conditions (Lagrange *et al.*, 1976; Wiegeshaus *et al.*, 1971). Any new recombinant vaccine must induce a longer-lived, memory immune T-cell response than any of the existent strains of BCG (Collins, 1991). At present, we know very little about the antigens (or genes) responsible for inducing this type of persistent immune memory response (Orme, 1988).

The ability of mice to inactivate *M. smegmatis* has been ascribed to the presence of the *Bcg^r* gene and seems to be independent of an acquired T-cell-mediated immune response (Denis *et al.*, 1990). These investigators reported substantial survival by *M. smegmatis* in peritoneal and splenic macrophages isolated from B10.A mice lacking the *Bcg^r* gene. In the present study, C57BL/6 (*Bcg^s*) mice rapidly inactivated both the r*M. smegmatis* and its vector

Fig. 6. Viable counts of lung homogenates from C57BL/6 mice vaccinated, at the times indicated by arrows, with two doses of 20 µg (10^8 acid fast bacilli) heat-killed r*M. smegmatis* J3R suspended in 0.2 ml Freund's incomplete adjuvant and challenged aerogenically 3 weeks later with approximately 10^3 *M. tuberculosis* (▲). Controls received Freund's incomplete adjuvant alone (■), or 20 µg of the vector control in Freund's incomplete adjuvant (●) or 10^6 c.f.u. BCG Pasteur (□).

control and survival of the recombinant in the corresponding macrophage cultures was equally short-lived (Figs. 3 and 4). Despite the mycobactericidal activity shown by the macrophage monolayer, this assay may still constitute a suitable screening procedure for the detection of *in vivo* growth (*avg*) genes (Pascopella *et al.*, 1994) transferred from other mycobacterial species. The *in vivo* infection protocol has the additional technical advantage that phagocytosis occurred more efficiently at lower multiplicities of infection, resulting in fewer extracellular mycobacteria within the macrophage culture. The presence of gentamicin in the culture medium to prevent overgrowth by non-cell-associated organisms may explain the decreased survival of *M. smegmatis* noted in the present study compared to that reported in the earlier study (Denis *et al.*, 1990).

In the present study, the BCG genomic library was expressed in *M. smegmatis*, which has long been known to be completely avirulent for both mice and people (Collins, 1983). Even when introduced into the tissues in massive numbers, the recombinant was inactivated before it could induce a detectable cell-mediated immune response to the transferred BCG antigens. The only way to achieve protection with this recombinant was to suspend the organisms in Freund's adjuvant administered as two doses over a 3 week interval. Under these experimental conditions, acquired resistance to the continued growth of the virulent *M. tuberculosis* challenge within the lungs of the J3R vaccinated mice was observed, a finding consistent with earlier protection studies carried out using this type of adjuvanted vaccine (Collins & Mackaness, 1970b; Hubbard *et al.*, 1992). Although the difference in

viable numbers of H37Rv in the lungs of the J3R versus vector control vaccinated mice was modest, it was statistically significant ($P < 0.01$) and comparable to that achieved using live BCG vaccine (Fig. 6). This difference correlated with increased survival by the J3R-vaccinated mice, none of which died during the 12 week challenge period, while all of the vector controls succumbed to the aerogenic challenge. The need for this type of adjuvant renders the present vaccine of little practical value for use in humans, but the mere fact that it worked at all makes this recombinant worth investigating further.

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