



Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis ☆

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☆ This work was funded by the European Community (QKL2-CT1999-01093) and the Department of Health, UK.

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Accepted 17 September 2004

KEYWORDS

Vaccine;
Tuberculosis;
Guinea pig;
Aerosol

Summary The TB Vaccine Cluster project funded by the EU Fifth Framework programme aims to provide novel vaccines against tuberculosis that are suitable for evaluation in humans. This paper describes the studies of the protective efficacy of vaccines in a guinea pig aerosol-infection model of primary tuberculosis. The objective was to conduct comparative evaluations of vaccines that had previously demonstrated efficacy in other animal models. Groups of 6 guinea pigs were immunized with vaccines provided by the relevant EU Vaccine Cluster partners. Survival over 17 or 26 weeks was used as the principal measure of vaccine efficacy following aerosol challenge with H37Rv. Counts of mycobacteria in lungs and spleens, and histopathological changes in the lungs, were also used to provide evidence of protection.

A total of 24 vaccines were evaluated in 4 experiments each of a different design. A heterologous prime-boost strategy of DNA and MVA, each expressing Ag85A and a fusion protein of ESAT-6 and Ag85B in adjuvant, protected the guinea pigs to the same extent as BCG. Genetically modified BCG vaccines and boosted BCG strategies also protected guinea pigs to the same extent as BCG but not statistically significantly better. A relatively high aerosol-challenge dose and evaluation over a protracted time post-challenge allowed superior protection over BCG to be demonstrated by BCG boosted with MVA and fowl pox vectors expressing Ag85A.

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Introduction

Tuberculosis (TB) kills in excess of 2 million people every year and the global epidemic is increasing. The current and only TB vaccine, *Mycobacterium bovis* bacille Calmette–Guérin (BCG), has been applied world-wide for several decades but possesses many drawbacks,¹ including variable efficacy in humans, an inability to protect against re-activation or re-infection, and pathogenicity in the immunocompromised host. Thus, there have been concerted efforts towards the discovery and development of a vaccine to replace BCG. Publication of the complete *M. tuberculosis* genome² and developments in both laboratory and 'in silico' methods of screening large numbers of proteins for immunogenicity has now accelerated the process of antigen discovery. In addition to the growing number of potential antigens, novel adjuvants and delivery systems are continually being developed.

The TB Vaccine Cluster project funded by the European Union Fifth Framework programme aimed to develop novel vaccines against tuberculosis that would be superior to BCG and suitable for evaluation in humans. This included a step-wise evalua-

tion of new candidate vaccines in mice, guinea pigs and non-human primates. Mouse studies enabled the initial selection of promising candidates by measuring the immunogenicity and protection against virulent challenge, and these candidates were then evaluated using the more discriminative aerosol-infection guinea pig model. Criteria for the selection of candidates for evaluation in the guinea pig model included evidence of immunogenicity and protective efficacy equivalent to or better than BCG in mouse and in some cases guinea pig models. The programme provided an opportunity to directly compare, within a single experiment, a number of vaccine candidates which had previously been demonstrated to be efficacious when evaluated separately. Here, we report the results of these comparative studies.

Materials and methods

Vaccines

The vaccines evaluated in the studies are listed in Table 1. The source of each vaccine is given and,

Table 1 Details, and sources of vaccines evaluated in the EU studies.

Vaccine	Short name	Source and references
Experiment 1		
Heterologous prime-boost of DNA and MVA both expressing Ag85A	DNA prime MVA/Ag85A boost	Prof. A.V.S. Hill, ¹⁴ Oxford, UK
Ag85B/ESAT-6 fusion protein (Hybrid 1) in DDA/MPL [®]	Hybrid 1 in DDA/MPL [®]	Prof. P. Andersen, SSI, Copenhagen, Denmark ⁷
Hybrid 1 protein in ASO2 adjuvant	Hybrid 1 in ASO2	Prof. P. Andersen, GSK (Rixensart, Belgium) for adjuvant
Plasmid DNA-encoding Hsp65 (CMV4.65)	Hsp65 DNA	Prof. D. Lowrie, ¹⁵ NIMR, London, UK
Arabinomannan (AM)—Mycobacterial protein Ag85B conjugate in L3 adjuvant	AM–Ag85B conjugate in L3	Prof. S.B. Svenson, SMI, Stockholm and SLU, Uppsala, Sweden ^{16–18}
Experiment 2		
Ag85A recombinant protein in ASO2 adjuvant	rAg85A protein in ASO2	Prof THM Ottenhof, ¹⁹ Leiden, The Netherlands; GSK for adjuvant
Fowlpox-Ag85A and MVA-Ag85A prime-boost Ag85A DNA in vaxfectin	Fowlpox/Ag85A–MVA/Ag85A Ag85A DNA in vaxfectin	Prof. A.V.S. Hill Dr. K. Huygen, ²⁰ Brussels, Belgium
Ag85A DNA in vaxfectin with rAg85A protein in ASO2 boost	Ag85A DNA in vaxfectin, rAg85A in ASO2 boost	Dr. K. Huygen
Hybrid 1 protein in DDA/MPL [®] +trehalose dibehenate (TDB)	Hybrid 1 protein in DDA/MPL [®] /TDB	Prof. P. Andersen
Hybrid 1 protein in DDA/MPL [®] , sub-cutaneous prime, boosted orally with Hybrid 1 in TDB	Hybrid 1 protein, oral boost	Prof. P. Andersen
Hybrid 1–AM conjugate in L3	Hybrid 1–AM in L3	Prof. S.B. Svenson and Prof. P. Andersen
Experiment 3		
<i>M. tuberculosis</i> <i>trpD</i> [−] mutant	<i>M. tuberculosis</i> <i>trpD</i> [−] mutant	Prof. N.G. Stoker, ^{21,22} London, UK
rBCG-expressing listeriolysin (Hly)	rBCG-Hly	Prof. S.H.E. Kaufmann, MPIIB, Berlin, Germany ²³
BCG Pasteur::RD1-2F9	rBCG-RD1	Prof. S. Cole, ⁸ IP, Paris, France
BCG Danish 1331, prime+boost with hybrid 1 in DDA/MPL [®]	BCG+hybrid 1 in DDA/MPL [®]	Prof. P. Andersen
BCG Danish 1331 prime+boost with MVA expressing Ag85A	BCG+MVA/Ag85A	Prof. A.V.S. Hill
BCG Danish 1331+BCG Danish 1331	BCG+BCG	SSI, Copenhagen, Denmark
Experiment 4		
<i>M. tuberculosis</i> <i>phoP</i> [−] mutant	<i>M. tuberculosis</i> <i>phoP</i> [−] mutant	Prof. B. Gicquel Paris, France; Prof. C. Martin, Zaragoza, Spain ^{9,24}
PstS3–Ag85A DNA prime–BCG Danish 1331 boost	PstS3–Ag85A DNA–BCG	Dr. K. Huygen
<i>M. microti</i> OV254::RD1-2F9	<i>M. microti</i> -RD1	Prof. S. Cole ¹⁰
BCG Danish 1331+MVA/85A+FP/85A	BCG+MVA/85A+FP/85A	Prof. A.V.S. Hill ¹¹
r Δ <i>ureC</i> BCG expressing Hly	r <i>ureC</i> BCG expressing Hly	Prof. S.H.E. Kaufmann
AM–Tetanus toxoid, conjugate in L3 adjuvant	Tetanus toxoid-AM+L3	Prof. S.B. Svenson

where relevant, supporting background information is referenced. Four experiments were conducted and the candidates are listed in experiment groups. The grouping of the candidates in each experiment reflects the aim to compare similar types of vaccine e.g. sub-units or live attenuated, although it was not always possible to design the experiment where one type of vaccine was tested exclusively. A selection process was established in which vaccines had to satisfy criteria that would show a potential for the vaccine to be effective against challenge with virulent *M. tuberculosis*. These criteria included immunogenicity and/or protection in mice or guinea pigs.

The candidates in Experiments 1 and 2 were selected in order to compare sub-unit vaccines in different delivery systems and were predominantly based on two antigens, antigen 85A (Ag85A) and a fusion protein between antigen 85B and ESAT-6 (referred to throughout as 'Hybrid 1'). A number of different delivery systems were used, including recombinant or native protein in various adjuvants, recombinant pox viruses and plasmid DNA. Combinations of delivery systems and routes of administration of the antigen were also tested.

The candidates in Experiments 3 and 4 were predominantly live attenuated vaccines. This included targeted mutants of *M. tuberculosis* and genetic modifications of BCG; for these, an additional requirement for selection was the demonstration of attenuation in immunocompetent and/or immunocompromised mice. Also included in these studies were vaccine candidates given in addition to conventional BCG vaccination.

Immunization

The immunization schedules for each candidate were specified by the provider of the vaccines and are briefly described in Table 2. The vaccines were supplied as prepared formulations wherever possible and, for those vaccines that required formulation immediately prior to inoculation, detailed instructions were provided and followed.

Female Dunkin–Hartley guinea pigs (weighing between 250 and 300 g, and free of infection) were obtained from a commercial supplier (David Hall, Burton-on-Trent, UK). Groups of 6 guinea pigs were used to evaluate the efficacy of each candidate vaccine compared with BCG and saline controls. Individual animals were identified using sub-cutaneously implanted microchips (PLEXX BV, The Netherlands). Animals were vaccinated as appropriate and then allowed to rest for at least 6 weeks prior to aerosol challenge with *M. tuberculosis*.

Rationale for challenge doses

In Experiment 1, the intention was to infect the animals with a relatively low dose (10–50 cfu) aerosol challenge since this is within the range of doses conventionally used in guinea pig models of tuberculosis. In Experiments 2–4 the challenge dose was increased with the intention to make the model more stringent and thus discriminate vaccine candidates that had already been proven to be efficacious in previous studies.

Aerosol challenge with *M. tuberculosis*

Aerosol challenge was performed using a contained Henderson apparatus as previously described.^{3,4} Fine particle aerosols of *M. tuberculosis* H37Rv, with a mean diameter of 2 µm, (diameter range, 0.5–7 µm)⁵ were generated using a Collison nebulizer and delivered directly to the animal snout. The aerosol was generated from a saline suspension containing 5×10^6 organisms per ml in order to obtain an estimated retained, inhaled dose of approximately 10–50 colony forming units (cfu)/lung, (Experiment 1) or from a suspension containing 5×10^7 or 1×10^8 cfu/ml in order to deliver 500 or 1000 cfu to the lungs (Experiments 2–4). The Henderson apparatus allows controlled delivery of aerosols to the animals and the reproducibility of the system and relationship between inhaled cfu and the concentration of organisms in the nebulizer have been described previously.^{3,6}

Measurement of protective efficacy

Animals were monitored regularly for weight changes as indicators of disease, and were killed humanely at 17 weeks (Experiments 1–3) or 26 weeks (Experiment 4) post-challenge or at the humane endpoint (20% loss of maximal body weight). Protection was primarily assessed by comparing the overall survival time of the vaccinated groups of animals with the control groups (BCG and un-vaccinated) over the period of the experiment. Animals killed at the humane endpoint were considered to have died due to infection for the purposes of analysing survival. The vaccine was considered to have offered protection if the survival time of the group was equivalent to or significantly longer than that of the BCG control group, and was significantly longer than the saline control group.

The extent of pulmonary disease and disseminated infection as measured by total lung consolidation and by comparison of lung and spleen

Table 2 Vaccination schedules for all the experiments.

Vaccine	Vaccination schedule	
	Quantity, volume, route and number of inoculations	Vaccine-challenge interval* (weeks)
Experiment 1		
DNA prime MVA/Ag85A boost	2 × 200 µg DNA i.m., 2 × 10 ⁷ p.f.u. MVA, i.d., 2 weeks apart	6
Hybrid 1 in DDA/MPL [®]	3 × 20 µg, s.c. 3 weeks apart	6
Hybrid 1 in ASO2	3 × 20 µg, s.c. 3 weeks apart	6
Hsp65 DNA	3 × 100 µg, i.m. 3 weeks apart	6
AM–Ag85B conjugate in L3	15 µg carbohydrate, 10 µg protein per dose, given s.c. once then i.n. 3 weeks later.	6
Experiment 2		
rAg85A protein in ASO2	3 × 20 µg, s.c., 3 weeks apart	6
Fowlpox/Ag85A–MVA/Ag85A	FP, followed by MVA, FP, MVA, all given i.d. at 10 ⁷ p.f.u., 2 weeks apart	6
Ag85A DNA in vaxfectin	3 × 100 µg DNA i.m., 3 weeks apart	6
Ag85A DNA in vaxfectin, rAg85A in ASO2 boost	2 × 100 µg DNA, i.m., 1 × 20 µg s.c., protein, 3 weeks apart	6
Hybrid 1 protein in DDA/MPL [®] /TDB	3 × 20 µg s.c., 3 weeks apart	6
Hybrid 1 protein, oral boost	1 × 20 µg s.c, 2 × 20 µg oral, 3 weeks apart	6
Hybrid 1–AM in L3	15 µg carbohydrate, 10 µg protein per dose given s.c. once and x2 i.n., 3 weeks apart	6
Experiment 3		
<i>M. tuberculosis</i> trpD ⁻ mutant	5 × 10 ⁴ cfu s.c. 12 weeks pre-challenge, animals housed at ACDP containment level 3.	12
rBCG-Hly	5 × 10 ⁴ cfu s.c. 12 weeks pre-challenge	12
rBCG-RD1 region	5 × 10 ⁴ cfu s.c. 12 weeks pre-challenge	12
BCG+Hybrid 1 in DDA/MPL [®]	5 × 10 ⁴ cfu s.c. BCG (1331), 20 µg protein 4 weeks later	8
BCG+MVA/Ag85A	5 × 10 ⁴ cfu s.c. BCG (1331), 10 ⁷ p.f.u. MVA/85A 4 weeks later	8
BCG+BCG	5 × 10 ⁴ cfu s.c. BCG (1331) x2, 4 weeks apart	8
Experiment 4		
PstS3–Ag85A DNA–BCG	2 × 100 µg DNA i.m., 2 weeks apart, 5 × 10 ⁴ cfu s.c. BCG (1331) 4 weeks later.	10
<i>M. microti</i> –RD1	5 × 10 ⁴ cfu s.c. 10 weeks pre-challenge, animals housed at ACDP containment level 3.	10
BCG+MVA/85A+FP/85A	5 × 10 ⁴ cfu s.c. BCG (1331), 10 ⁷ p.f.u. MVA/85A 4 weeks later, 10 ⁷ p.f.u. FP9/85A, 2 weeks later.	6
rΔ <i>ureC</i> BCG expressing Hly	5 × 10 ⁴ cfu s.c. 10 weeks pre-challenge	10
Tetanus toxoid-AM+L3	1 × 100 µl s.c., 2 × 50 µl i.n., 3 weeks apart	8

s.c. = sub-cutaneous, i.n. = intranasal, p.f.u. = plaque forming units, i.m. = intramuscular, i.d. = intradermal.

* = interval between administration of final inoculation of vaccine and aerosol challenge.

cfu were also used to compare the vaccinated animals with the control groups. A significant reduction in any of these parameters in the vaccinated animals when compared with the control groups was considered as a protective effect of the vaccine.

Agar infusion of lungs

To improve the accuracy of image analysis techniques to measure consolidation, a controlled inflation of lungs was performed. An agar infusion technique was used in order to maintain the ability

to culture lung lobes for viable *M. tuberculosis*. Guinea pigs were killed by intraperitoneal injection of pentobarbitone (Euthatal) and sterile molten agar (2%) was injected into the lungs via an incision in the trachea (exposed in the neck) whilst the thorax was still intact. The trachea was then clamped and the lungs were dissected once the agar had set. Three lung lobes (right caudal, right middle and accessory lobes) were removed and fixed in 10% neutral-buffered formaldehyde. These were cut at the widest organ diameter, ensuring each lobe was sectioned at the same position for every animal, embedded in paraffin wax blocks and 5 µm sections cut on to glass slides. Each lung section was stained with Haematoxylin & Eosin (H&E) for objective measurement of consolidation by image analysis. The remaining lung lobes were aseptically removed for bacteriology (cfu counts).

Image analysis of lung sections

An image of each H&E-stained section of agar-infused lung was captured using Image Analysis equipment (Zeiss Axio Cam digital camera connected to a Zoom Micro-NIKKOR 55 mm 1:2.8 photo lens using a MicroCam C-mount converter 0.65 × to Nikon F-bayonet) and Axio-Vision 2.0.5 software and archived using the Image Access 3.10 software. Analysis of the images was undertaken using the Zeiss KS300 3.0 software. A software programme was written to assess the area of consolidation of each lobe objectively. This measured the area of each lung lobe and the area of consolidation, and thus calculated the percentage area of consolidation. Mean percentages of consolidation per animal (3 lobes per animal) and per vaccine group were determined.

Bacterial counts in organs

Tissues were homogenized in 10 ml (lungs) or 5 ml (spleens) of sterile distilled water using a rotating blade macerator system. Viable counts were performed on the macerate by preparing decimal dilutions in sterile deionized water and plating 100 µl aliquots onto Middlebrook 7H11+OADC agar. Plates were incubated at 37 °C for 3 weeks before counting the number of *M. tuberculosis* colonies (cfu).

Statistical analyses

Statistical analyses were performed using Minitab (version 13.32). The ability of vaccines to prolong

survival was compared with controls using Kaplan–Meier survival estimates with right censoring only. A Log Rank distribution analysis was used to identify statistical significance between control groups and individual test vaccine groups. The cfu and consolidation data were analysed by ANOVA, using Fishers pairwise comparisons to compare mean values of the vaccine groups with either the saline or BCG control groups.

Results

A summary of the results of all the experiments is shown in Table 3.

Experiment 1

A Kaplan–Meier plot of the survival data for Experiment 1 is shown in Fig. 1. Over the 17 week period of the study, several of the animals in the saline control and test vaccine groups became ill and were killed at the humane end-point. Two of the vaccine candidates, the DNA/MVA-Ag85A prime-boost candidate and Hybrid 1 in DDA/MPL^{®7} prolonged survival significantly ($p = 0.03$ and 0.025 , respectively) longer than the saline control and equivalent to the BCG control (Fig. 1). None of the vaccines showed improved survival over the BCG control.

All of the vaccines reduced numbers of viable *M. tuberculosis* (cfu) in the spleens but no significant protection was observed in the lungs (Table 3). The group of animals vaccinated with the DNA/MVA-Ag85A prime-boost candidate also had a significantly lower mean percentage lung consolidation than the saline controls (Table 3).

Experiment 2

The challenge dose in Experiments 2 was approximately 100-fold higher than Experiment 1 and as a result, the time taken for severe disease to develop in the unvaccinated controls was reduced and by the end of the 17 week period of the experiment, none of the animals in this group had survived. In contrast, all of the BCG-vaccinated animals survived to the end of the experiment. However, none of the vaccines tested in Experiment 2 showed a statistically significant improved survival over the saline control although Hybrid 1 (s.c. prime, oral boost) significantly reduced CFU in spleens (Table 3).

Table 3 Summary of the results of all the vaccine evaluations indicating whether each candidate demonstrated a statistically significant effect compared with control groups.

Vaccine	Significant protection as demonstrated by:			
	Prolonged survival	Reduced cfu in lungs	Reduced cfu in spleens	% lung consolidation
Experiment 1				
DNA prime MVA/Ag85A boost	✓	x	✓	✓
Hybrid 1 in DDA/MPL [®]	✓	x	✓	x
Hybrid 1 in ASO2 adjuvant	x	x	✓	x
Hsp65 DNA	x	x	✓	x
AM–Ag85B conjugate in L3	x	x	✓	x
BCG control	✓	✓	✓	✓
Experiment 2				
rAg85A protein in ASO2	x	x	x	NT
Fowlpox/Ag85A–MVA/Ag85A prime-boost	x	x	x	NT
Ag85A DNA in vaxfectin	x	x	x	NT
Ag85A DNA in vaxfectin boosted with rAg85A protein in S2	x	x	x	NT
Hybrid 1 protein in DDA/MPL [®]	x	x	x	NT
Hybrid 1 protein, sub-cutaneous prime, oral boost	x	x	✓	NT
Hybrid 1–arabinomannan conjugate in L3	x	x	x	NT
BCG control	✓	✓	✓	NT
Experiment 3				
<i>M. tuberculosis trpD</i> ⁻ mutant	x	x	x	NT
rBCG-Hly	✓	✓	✓	✓
rBCG-RD1 region	✓	✓	✓	✓
BCG+Hybrid 1 in DDA/MPL [®]	✓	✓	✓	✓
BCG+MVA/Ag85A	✓	✓	✓	✓
BCG+BCG	✓	✓	✓	✓
BCG control	✓	✓	✓	✓
Experiment 4				
PstS3–Ag85ADNA–BCG	✓	✓	✓	✓
<i>M. microti</i> –RD1 region	✓	✓	✓	✓
BCG+MVA/85A+FP/85A	✓✓	✓	✓	✓
rΔ <i>ureC</i> BCG expressing Hly	✓	✓	✓	✓
Tetanus toxoid-arabinomannan+L3	x	x	x	x
BCG control	✓	✓	✓	✓

✓ = significantly better protection than the saline control ($p = <0.05$).

✓✓ = significantly better protection than the BCG control ($p = <0.05$).

x = no protection.

NT = not tested.

Experiments 3 and 4

Animals in Experiment 3 were challenged with approximately 1000 cfu *M. tuberculosis* by aerosol and the resulting disease in the unvaccinated guinea pigs was similar to that observed in Experiment 2 i.e. there were no survivors at the end of the 17 week post-challenge period. The vaccines based on BCG (rBCG-Hly, rBCG-RD1,⁸

BCG+Hybrid1, BCG+MVA/85A, BCG+BCG) all prolonged survival significantly longer than the saline control but over the 17 weeks post-challenge period, it was not possible to discriminate between these vaccines and the BCG control. Similarly, the vaccines that prolonged survival also significantly reduced both cfus in organs and lung pathology but did not show a statistically significant improvement over the BCG control group (Table 3).

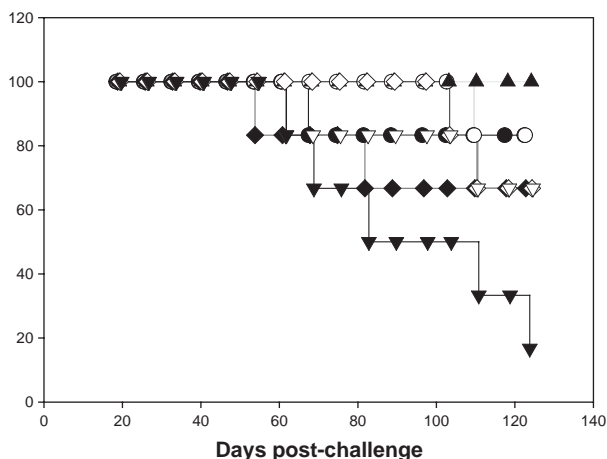


Figure 1 Survival of vaccinated guinea pigs following aerosol infection with *M. tuberculosis*. Experiment 1, guinea pigs given low-dose aerosol challenge with H37Rv and monitored for 124 days. Vaccine groups: ● DNA+MVA/85A, ○ Hyb1 in DDA/MPL[®], ◇ Hyb1 in ASO2, ▽ DNA-Hsp65, Protein-AM conjugate, ▲ BCG control, ▼ Saline control.

The vaccines in Experiment 4 were mostly live attenuated and the study design was changed compared with Experiment 3 with an aim to identify any vaccines with improved efficacy over BCG. Vaccinated animals were challenged with a high aerosol dose (approx. 500 cfu) and were followed for up to 26 weeks post-challenge. Over this period, all of the animals in the unvaccinated control group succumbed to infection and between weeks 17 and 26 post-challenge, four of the animals in the BCG control group were killed at the humane end-point. The results of the group vaccinated with the *M. tuberculosis* phoP mutant will be reported elsewhere.⁹ Three of the vaccines, PstS3-Ag85A DNA+BCG, *M. microti*-RD1¹⁰ and $r\Delta$ *ureC* BCG-Hly prolonged survival compared with the unvaccinated controls but the statistical analysis of the survival data did not indicate a significant improvement over the BCG control. In the group vaccinated with BCG boosted by MVA and Fowlpox vectors expressing Ag85A¹¹ there were no deaths over the experiment period. Analysis of survival times showed that this group had significantly prolonged survival compared with the BCG controls ($p = 0.018$).

Discussion

The aim of these studies was to screen a number of potential TB candidate vaccines in a stringent guinea pig aerosol infection model of primary

tuberculosis. A total of 24 candidates were tested and several of these demonstrated a protective efficacy equivalent to the current BCG vaccine, which was included in all experiments as a positive control. The ability of the test vaccine to prevent severe illness was used as the primary indicator of protective efficacy and this was measured by an analysis of survival over the period of the experiment. Two sub-unit vaccines, a heterologous prime-boost strategy of DNA and MVA each expressing Ag85A and a fusion protein consisting of ESAT-6 and Ag85B (Hybrid 1),⁷ protected the guinea pigs to the same extent as BCG by prolonging survival following low-dose aerosol challenge as compared with the unvaccinated controls. Against a high-dose aerosol challenge, eight vaccine candidates gave a level of protection equivalent to the BCG control. These were either genetically modified non-pathogenic mycobacteria, or were prime-boost strategies involving vaccines that were given in addition to conventional BCG (Danish 1331). The genetically modified strains were: rBCG expressing listeriolysin, BCG Pasteur::RD1-2F9,⁸ *M. microti* OV254::RD1-2F9¹⁰ and $r\Delta$ *ureC* BCG expressing listeriolysin. The prime-boost strategies were: BCG followed by Hybrid 1 in DDA/MPL[®], BCG followed by MVA expressing Ag85A, BCG followed by BCG and DNA encoding both PstS-3 and Ag85A followed by BCG. All of the candidates that demonstrated a protective efficacy equivalent to the BCG control would be considered as having the potential to be taken forward for further improvement and evaluation in other test systems such as non-human primates or animal models of post-primary disease. Other factors such as previous data obtained in different animal models will influence the decision to progress vaccines into potentially expensive further trials and it will also be important to establish that key experimental results in animal models are reproducible.

Ideally, the outcome of a programme of work to identify a vaccine that could replace or improve upon BCG would be a demonstration in animal models that the new vaccine candidate had significantly better protective efficacy than BCG. In the guinea pig model this has been demonstrated by an ability of the candidate vaccine to prevent replication of *M. tuberculosis* in the lungs to a significantly greater extent than the BCG control.¹² BCG vaccination will protect guinea pigs against low-dose challenge with *M. tuberculosis* such that they survive for one or two years post challenge¹³ and there are thus practical difficulties in demonstrating that a candidate vaccine can significantly improve upon this. In this study we employed a strategy of using a high-dose challenge in order to

make the test system more stringent. Over a period of 17 weeks (Experiment 3), it was not possible to identify differences in the test vaccines versus BCG but when the period of study was extended to 26 weeks, the BCG-vaccinated animals had begun to be overwhelmed by the higher challenge dose. In contrast, animals vaccinated with BCG boosted with MVA and fowlpox vectors expressing Ag85A¹¹ significantly prolonged survival of the animals compared with the BCG control.

The high challenge doses used in these experiments resulted in a disease model that was much more severe than that conventionally used to evaluate the protective efficacy of TB vaccines. However, since the aim of these studies was to discriminate vaccine candidates which had already been proven to be effective in other test models, it was important that the system should be stringent. It was apparent from these studies that the high challenge dose is best applied to vaccines that are based on or are similar to BCG (i.e. live attenuated). For some of the vaccines, the system was probably too stringent and a failure to offer protection equivalent to BCG in the high-dose model should not indicate that the vaccine is ineffective but rather that it should be re-evaluated in a low-dose challenge system over a prolonged post-challenge period.

The 4 experiments reported here were each of a different design, with challenge doses and length of post-challenge period being the main variables. Thus the protective efficacy of a vaccine candidate can be compared only with the other vaccines in that experiment and in relation to the positive and negative controls. The changes to the experimental design were influenced strongly by the main objective of these studies which was to discriminate between the vaccine candidates and to identify the most promising of all those tested within a programme that was limited by both time and budget. Thus, some of the vaccines may have performed better (or worse) when tested in a different study protocol. As an example, it is possible that some of the vaccines in Experiment 3 could have demonstrated a superior protective efficacy to BCG if the post-challenge period had been extended from 17 to 26 weeks. It is important that the design of future TB vaccine evaluations in guinea pigs takes into account the potential for variability in vaccine potency which can be caused by differences in experimental schedules. It is also necessary to ensure that there is comparability with previous studies conducted within the EC programme and with similar vaccine evaluation programmes conducted elsewhere.

Enumeration of viable *M. tuberculosis* (cfu) in the lungs and spleens of all animals in these studies revealed that the cfu in animals that had been killed at the humane end-point were significantly higher than cfu in animals that survived to the end of the experiment. This is perhaps not surprising but the practical outcome was a large spread in the cfu values within vaccine groups comprised of both killed and surviving animals. Thus, because in these studies survival was the principal read out of vaccine efficacy, cfu in organs was not always an informative measure of vaccine efficacy.

In conclusion, the vaccine evaluation studies reported here represent an objective evaluation of a number of TB vaccine candidates which allowed the identification of several promising vaccine strategies to take forward to future development and evaluation and thus progression to human clinical trials. An EC Sixth Framework-funded programme of work which began in 2004 will provide a means of such progression, and the evaluations in guinea pigs will remain in this programme as an important stage in the pre-clinical development of potential vaccines.

Acknowledgements

This work was funded by the European Community (QKL2-CT1999-01093) and the Department of Health (UK). The views expressed in this publication are those of the authors and not necessarily those of the funding bodies. All the providers of the vaccines, as listed in Table 1 are gratefully acknowledged, as are members of the TB Cluster Steering Committee, namely Prof. Brigitte Gicquel, Prof. Douglas Young, Prof. Stefan H.E. Kaufmann, Prof. THM Ottenhoff, Dr. Jelle Thole, Dr. Carlos Martin, Dr. Jean-Jacques Fournie and Prof. Peter Andersen. The staff in the Biological Investigations Group at HPA Porton Down are sincerely thanked for their technical support.

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