MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

Date project completed:
31/03/1999

Research and Development

Final Project Report

(Not to be used for LINK projects)

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Section 1 : Identification sheet

1.	(a)	MAFF Project Code	SE3202
	(b)	Project Title	The development of animal models to test candidate vaccines for *M. bovis* infection in badgers
	(c)	MAFF Project Officer	Dr Fiona Stuart
	(d)	Name and address of contractor	Veterinary Laboratories Agency Weybridge, Woodham Lane, New Haw, Addlestone, Surrey Postcode KT15 3NB
	(e)	Contractor's Project Officer	Dr R. G. Hewinson
	(f)	Project start date	01/04/1998 — Project end date 31/03/1999
	(g)	Final year costs: **approved** expenditure	£202,445
		actual expenditure	£183,168
	(h)	Total project costs / total staff input: **approved** project expenditure	£202,445
		actual project expenditure	£183,168
		***approved** staff input	2.626
		***actual** staff input	1.881
	(i)	Date report sent to MAFF	10/12/1999
	(j)	Is there any Intellectual Property arising from this project ?	NO

***staff years of direct science effort**

01	30/12/1997	To establish S.O.P. for antigen-specific badger lymphoproliferation
02	30/11/1997	To establish *M. bovis* challenge model in mice.
03	30/04/1998	To establish *M. bovis* challenge model in guinea pigs
04	30/05/1998	To compare two *M. bovis* isolates for virulence in guinea pigs
05	30/06/1998	Ability of BCG auxotrophs to protect against challenge and sensitise guinea pigs to tuberculin tested
06	30/01/1999	Ag85 DNA and MPB83 DNA vaccine protection studies completed in guinea pigs
07	31/05/1998	To sequence the gene encoding badger IFNγ
08	31/08/1998	To purify badger IgA
09	31/03/1999	DNA vaccination protection studies completed in mice
10	31/12/1998	Assess the feasibility of carrying out vaccine evaluation in badgers at CNEVA, Nancy, France
11	31/03/1999	TGF-β, TNF-α and IFN-γ assays developed for guinea pigs
12	31/03/1999	Annual progress report and publications produced and final report prepared

. List the primary milestones for the final year.

It is the responsibility of the contractor to check fully that ALL primary milestones have been met and to provide a detailed explanation if this has not proved possible

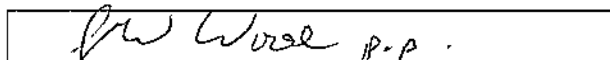
Milestones		Target date	Milestones met?	
Number	Title		in full	on time
03/03	Parameters of guinea pig aerosol challenge model determined in terms of challenge dose, mean survival time, and subsequent pathology *post mortem*.	30/04/1998	YES	YES
04/02	Guinea pigs challenged with low aerosol dose with each field strain of *M. bovis* and bacterial burden in kidneys and spleen determined.	30/05/1998	YES	YES
05/02	Guinea pigs challenged with a minimum of two auxotrophic strain(s) of BCG and parental strain BCG. Bacteria enumerated in the lungs and spleens of Guinea pigs killed at varying times after challenge.	30/06/1998	YES	YES
05/03	DTH response to intradermal PPD inoculation determined in animals challenged with a minimum of two auxotrophic strain(s) of BCG and parental strain BCG.	30/06/1998	YES	YES
06/01	Ability of DNA vaccine candidates Ag85 DNA and MPB83 DNA to protect guinea pigs from lethal aerosol challenge with *M. bovis* tested.	30/01/1999	YES	YES

Milestones		Target date	Milestones met?	
Number	Title		in full	on time
07/04	Entire IFNγ cDNA cloned and sequenced using badger-specific primers, PCR, and RACE. Sequences submitted to database.	30/06/1998	YES	YES
07/05	Badger IFNγ expressed using naked DNA in mice and used to raise monoclonal antibodies to the expressed product.	30/03/1999	YES	YES
08/04	Badger IgA purified using a sephadex G200 and/or JACALIN column.	30/06/1998	YES	YES
08/05	Any cross-reactivity of commercial anti-IgA antibodies to badger IgA determined.	31/08/1998	YES	YES
08/06	Badger IgA given to the Monoclonal Antibody Production Unit for raising a mAb.	31/08/1998	NO	NO
09/01	Mice immunised with DNA expression constructs Ag 85 DNA, MPB83 DNA and MPB70 DNA. Humoral and cellular immune response determined.	30/05/1998	NO	NO
09/02	Mice vaccinated with most (appropriately) immunogenic constructs and their ability to protect against *M. bovis* challenge determined.	31/03/1999	YES	YES
12/01	Submit annual report	31/05/1998	N/A	N/A
12/02	Four papers prepared: (1) Dev of an aerosol challenge model against *M. bovis* in guinea pigs; (2) Vaccination against *M. bovis* using BCG and auxotrophic strains of BCG; (3) Differences in virulence of M. bovis isolates in the guinea pig model; (4) DNA vaccines are expressed in the badger.	31/08/1998	YES	YES
12/03	Three papers prepared: (5) DNA vaccination with MPB83 protects against TB; (6) Antigen specific lymphocyte proliferation assays for badgers; (7) Cloning and sequencing of the gene encoding badger gamma interferon.	30/11/1998	YES	YES
12/04	Three papers prepared: (8) DNA vaccination against *M. bovis* infection in guinea pigs; (9) Differences in histopathology of *M. bovis* isolates in the guinea pig model; (10) Cloning and sequencing of guinea pig gamma interferon.	21/03/1999	NO	NO

If any milestones have not been met in the final year, an explanation should be included in Section 5.

Section 3 : Declaration

4. I declare that the information I have given in this report is correct to the best of my knowledge and belief. I understand that the information contained in this form may be held on a computer system.

Signature  Date 08.12.1999

Name Dr R.G. Hewinson

Position in Organisation Head, TB Research

Section 4 : Executive summary

The development of vaccines is a complex process requiring three areas of interlinked research. The development of candidate vaccines, testing the efficacy of those candidates against challenge in appropriate animal models, and analysis of the induced immune response in the animal model and target host. The overall objectives of this ROAME proposal were to establish relevant models with which to screen vaccine candidates for their ability to protect the host against *M. bovis* infection and to generate immunological reagents and procedures with which to study the immune response of badgers to *M. bovis* infection and vaccination.

An mouse model based on intravenous challenge with *Mycobacterium bovis* was developed to mimic bite wound challenge and used to identify a promising DNA vaccine (pCMV83) candidate based on the *M. bovis* antigen MPB83. BALB/c mice immunised with pCMV83 gave a mixed IgG_1/IgG_{2a} response to MPB83. Mice immunised with pCMV83 were protected from intravenous challenge with *M. bovis* (between 10^3 and 10^4 cfu) to a similar extent as those vaccinated with BCG.

A guinea pig aerosol challenge model was developed to mimic the natural, aerogenic route of *M. bovis* infection in cattle and badgers. This model was used to compare the virulence of the laboratory adapted strain, AN5, with two virulent *M. bovis* isolates. These isolates were found to vary in their ability to colonise the spleen and kidney. This model was also used to assess the protective efficacy of candidate DNA vaccine pCMV83. Ten weeks after low-dose *M. bovis* aerosol challenge, guinea pigs immunised with pCMV83 had 33% fewer lesions in their lungs compared with control animals but were not protected from haematogenous seeding of the spleen. The number of necrotic and mineralised pulmonary lesions in the guinea pigs was determined by X-ray and examination of fixed lungs *post mortem*. Lesions of control animals were predominantly mineralised. The opposite was seen in pCMV83-immunised animals where pulmonary lesions were predominantly necrotic. Compared with controls, BCG-vaccinated animals had an average of 1.4 log fewer *M. bovis* in their spleen (no bacteria were recovered from the spleens of 3/6 animals), and 74% fewer pulmonary lesions; of which equal numbers were mineralised and necrotic.

The guinea pig aerosol challenge model was also used to test the protective efficacy of two BCG auxotrophs mc^2798 and mc^2789 which have an absolute requirement for leucine and methionine, respectively. These auxotrophic strains differ from one another, and from BCG, in the length of time they persist in mice. The leucine auxotroph of BCG conferred significant protection to infection with both *M. bovis* and *M. tuberculosis* in the absence of a cutaneous hypersensitivity reaction to tuberculin. This result indicates that a new generation of vaccines based on BCG may be developed that are protective, safe for use in the immunocompromised, and do not preclude the use of the tuberculin skin test in both man and animals.

In order to assess the immune response to vaccination in guinea pigs and thereby identify possible correlates of protective immunity, lymphocyte proliferation, gamma interferon and TGF-β assays were developed for guinea pigs.

At the present time, experimental systems for vaccine evaluation in badgers are not available. One of the main requirements for such systems is the development of immunological assays and diagnostic tests so that selected vaccines could be tested in the badger. We therefore developed a lymphocyte transformation assay in order to detect cellular responses to vaccination and *M. bovis* infection in badgers. In addition, the badger gamma interferon gene was cloned and sequenced for subsequent development of a badger gamma interferon assay and badger IgA was isolated for future development of an assay to study local mucosal immunity in badgers.

Section 5 : Scientific report

Objective 01: Development of an antigen specific lymphocyte transformation assay (LTA) for badger

Since the immune response of the host to *M. bovis* infection is predominantly cellular rather than humoral, blood based T cell assays for badgers are likely to be important tools for epidemiological surveillance of *M. bovis* infection and for the development and evaluation of badger vaccines. An antigen specific LTA was developed for badgers which gave improved sensitivity over the BROCK TEST ELISA with similar specificity. This technology has been transferred to the Republic of Ireland to aid a study aimed at determining the immunological response of badgers vaccinated in the field with BCG. A paper describing the development of this assay has been published [D. Dalley et al., Vet Immunol. Immunopathol. (1999) 70, 85-94].

Methodology

Objective 02: Development of an intravenous (iv) *M. bovis* challenge model in mice

The parameters of infection for a murine M. bovis challenge model were determined. A paper describing this model and its use in determining the efficacy of a DNA vaccine (objective 9) has been accepted for publication in the journal 'Clinical Infectious Diseases' (formerly Reviews in Infectious Diseases).

Methodology

The *M. bovis* field isolates (5260/96), spoligotype '25', and (1692/96), spoligotype '17' were isolated from the pooled lymph nodes of M. bovis infected cattle in Staffordshire and Gloucester respectively. The *M. bovis* field isolates were propagated immediately prior to this study (i.e. passage 1) at 37 °C in Middlebrook 7H9 broth containing 4.16 g/l sodium pyruvate and 0.05% (v/v) Tween-80 supplemented with 10 % (v/v) Middlebrook ADC enrichment. Prior to challenge the inoculum of *M. bovis* was washed, resuspended in endotoxin-free PBS and dispersed by brief sonication using a Vibracell control unit plus CV18 converter fitted with a 3 mm diameter tip (Sonics & Materials Inc., Danbury, CT). Initially ten strains of mice were challenged intravenously with 10^5 cfu of *M. bovis*. This represented an inoculum two logarithms lower than that used routinely for intravenous challenge of mice with *M. tuberculosis* (i.e. 10^7 cfu). Subsequent studies to refine the challenge model used BALB/c mice and doses of 10^4 and 10^3 cfu *M. bovis* iv.

Results

The mouse model for infection with *M. bovis* was established by titrating doses of *M. bovis*. Initially, ten strains of mice were infected intravenously with the two different field isolates of *M. bovis* at a dose of 10^5 cfu. Both field isolates of *M. bovis* were considerably more virulent than reported for an equivalent dose of virulent strains of M. tuberculosis (including H37Rv, Erdman and a clinical isolate). Deaths occurred in the majority of mice from each strain before three weeks after challenge. *Post mortem* examination revealed severe tuberculosis affecting the lungs, spleen, liver and kidney. Greatest survival was seen with BALB/c and MRL/Lpr mutant mice. Subsequent studies to refine the challenge model used BALB/c mice and doses of 10^4 and 10^3 cfu *M. bovis* (5260/96) iv. At these doses all mice survived for seven weeks and had tuberculosis at *post mortem*. Lesions in lung and spleen were observed for both doses whereas kidney lesions were only observed in mice inoculated with 10^4 cfu *M. bovis*.

Conclusions

The testing of vaccine candidates in murine model for *M. bovis* infection has several advantages. Although mice are relatively insusceptible to tuberculosis in comparison with other mammalian species they are a cheap and convenient small animal model. Moreover, a large number of immunological reagents are available for mice so that the immune response elicited by both vaccination and challenge can be compared. In this study we established the parameters of an *M. bovis* challenge model so that vaccination-challenge experiments could be

performed. The use of this model to assess protective efficacy of DNA vaccines is described below under objective 9. The M. bovis field isolates from GB were found to be considerably more virulent for the mouse than reported for *M. tuberculosis*; causing death through acute tuberculosis. To overcome this, we had to use doses of *M. bovis* which were three to four logarithms less than the doses used in a similar study using a clinical isolate of *M. tuberculosis* in BALB/c mice (M. Jackson et al, 1999, Infection and Immunity, 67, 2867-73.). Lesions in the kidney were observed seven weeks after iv challenge with *M. bovis* at 10^4 but not 10^3 cfu. Thus a dose of 10^4 is required to generate a model of *M. bovis* kidney infection (which is thought to be responsible for the shedding of *M. bovis* via the urine in badgers).

Objective 03: Development of an *M. bovis* aerosol challenge model in guinea pigs

We have successfully defined the parameters of a guinea pig *M. bovis* aerosol challenge model in collaboration with the Pathogen Microbiology Division at CAMR, Salisbury.

Methodology

Female Dunkin-Hartley guinea pigs weighing between 350-450g were exposed for five minutes to bacterial aerosols containing particles mostly below 5μm diameter (diameter range 0.5-7μm, mean 2μm). The aerosol was generated from a suspension of *M. bovis* AN5 with a three-jet Collison nebulizer in conjunction with a modified version of the mobile Henderson apparatus. The apparatus allows controlled delivery of aerosols directly to the snouts of the animals without contamination of fur or eyes. Three doses were administered to groups of eight guinea pigs from suspensions containing approximately 10^6, 10^7 or 10^8 cfu/ml in order to obtain inhaled retained doses in the lungs of approximately 10, 100 and 1000 organisms, respectively. A control group of eight animals were exposed to a saline aerosol for five minutes.

For comparison, five female Dunkin-Hartley guinea pigs weighing between 350-450g were injected with 0.1μg wet mass of living *M. bovis* AN5 in a 500μl volume of saline in the flexor muscles of the crus region of the right hind leg.

Animals were monitored for clinical signs of disease and the experiment terminated when more than one animal died (5 weeks post challenge). Therefore the remaining animals were killed five weeks after infection by intracardiac overdose of sodium pentobarbitone. Examination was carried out immediately after death. External examination was followed by gross internal examination of the body condition, musculoskeletal system, neck region, thoracic and abdominal cavities. Retained bacterial dose in the lungs was estimated by enumeration of primary tubercle lesions at *post mortem*. The whole spleen and lungs were removed aseptically and placed separately into 5ml sterile distilled water for bacteriology.

Spleens and lungs were homogenised in 5ml sterile distilled water using a rotating blade macerator system. Viable counts were performed on the macerate by serial dilution in sterile distilled water and plating of dilutions onto Middlebrook 7H10 agar containing 4.16mg/ml sodium pyruvate and 10% (v/v) Middlebrook ADC enrichment. Plates were incubated at 37 ± 2^{0}C and examined after 4 weeks for growth of *M. bovis*. The number of colonies on each plate containing between 30 and 300 colonies was counted and recorded.

Results

Two animals died spontaneously before the end of the experiment: one in the 100 cfu aerosol group and one in the 1000 cfu aerosol group. Severe and extended gross lung lesions and lesions in the tracheobronchial lymph nodes were found in each at *post mortem*. The pathology was typical of tuberculosis; the assumed cause of death.

Lung lesions were present in all the guinea pigs infected via the aerosol route that survived until the end of the experiment. A number of lesions were round well-limited white foci of 2-4mm, many with central necrosis. These lesions most likely represent the primary sites of infection and were counted. Other lesions were smaller (0.5-1mm) white foci with more diffuse borders. These lesions probably represent metastatic (secondary) sites of infection. In contrast, the lung lesions in guinea pigs infected via the intramuscular route were small (0.5mm) white necrotic foci and with one exception, infrequent. These lesions were counted as primary sites of infection. Their smaller size most likely results from the extra time taken for the organisms to colonise the lung given their distal route of delivery. No lung lesions were evident in one animal.

Table 1 summarises the primary lesion counts from all guinea pigs. The mean number of primary lesions in the animals that received the two lower aerosol doses of *M .bovis* matched very closely our aimed retained dose of 10 and 100 organisms. We have found this to be reliably reproducible and suggests that each primary lesion arises from a single organism, or at least a single colony forming unit. At the highest dose (1000 cfu retained), the lung lesions formed coalescing foci of various sizes and shapes and it was impossible to count individual primary lesions. The intramuscular dose used in this study was approximately 100 cfu. In contrast to the same dose delivered via the respiratory route, the number of primary lesions in the lung was low and variable, with a mean count of six lesions per animal, and a median count of one.

TABLE 1. Number of primary lung lesions five weeks after infection with different retained doses of *M. bovis* AN5 via the aerosol or intramuscular route

	Animal								
Dose/Route	1	2	3	4	5	6	7	8	mean
saline control	0	0	0	0	0	0	0	0	0
10cfu/aerosol	16	17	27	15	8	13	12	7	14
100cfu/aerosol	66	87	122	†	122	81	101	84	95
1000cfu/aerosol	>176	>131	>160	>187	>287	†	>219	>152	>187
100 cfu/i.m.	1	26	1	0	1				6

† Animal died less than five weeks after infection.

Those animals infected intramuscularly exhibited gross pathology at the site of injection. Each animal had focal infiltrating necropurulent myositis of the right hind leg. Similarly, involvement of the draining lymph nodes was determined by the site of infection. For the animals infected via the respiratory route, this involved severe enlargement (up to 30mm in length) of the tracheobronchial lymph nodes with necrosis at their centre. The thymus and other lymph nodes of the thorax were also enlarged and necrotic. For the intramuscular route, reactive necropurulent lymphadenopathy involved the regional lymph nodes (deep inguinal and popliteal).

The spleen and liver of all animals contained white necrotic foci of 0.5-3mm diameter. The distribution of foci in the spleen was homogenous and subcapsular as well as internal. They were more numerous at the higher doses and more frequent in the animals infected via the respiratory route.

The observations made at *post mortem* were consistent with the bacterial burden found in the lungs and spleen of infected animals after culture of the tissue macerates. Table 2 summarises the bacterial counts obtained from the lungs of animals infected via the respiratory route. The increase in bacterial numbers over the five week period was 4-4.6 log and was independent of the retained dose. Ignoring numbers of bacteria killed by the animal, these figures are consistent with an *in vivo* doubling time of approximately 48 hours.

TABLE 2. Log cfu of bacteria in the lungs five weeks after infection with different retained doses of *M. bovis* AN5 via the aerosol route

	Animal								
Dose	1	2	3	4	5	6	7	8	mean
saline control	0	0	0	0	0	0	0	0	0
10cfu	ND	6.21	5.39	6.18	4.81	4.92	4.85	5.65	5.43
100cfu	5.80	5.55	5.48	†	7.43	5.28	5.81	6.68	6.00
1000cful	8.26	7.13	6.97	7.65	8.61	†	7.53	7.29	7.63

† Animal died less than five weeks after infection. ND Not done.

Table 3 summarises the bacterial counts obtained from the spleens of animals infected by either the respiratory or intramuscular route. The number of bacteria in the spleens of animals exposed to aerosolised *M. bovis* was lower than that of the lung. It is likely that this difference in bacterial load reflects the kinetics of haematogenous spread from the lungs to the spleen and other non-pulmonary tissues. The extent of spleen colonisation was not simply related to the retained dose delivered to the lungs. For retained doses of 10 and 100 cfu, exactly one log increase in spleen bacterial numbers was observed. However, increasing the retained dose a further log to 1000 cfu did not result in an increase in the number of organisms recovered from the spleen. Colonisation of the spleen by organisms introduced via the muscle was over 1.5 log lower than that observed following administration of the same dose of organisms to the lungs.

TABLE 3. Log cfu of bacteria in the spleen five weeks after infection with different retained doses of *M. bovis* AN5 via the aerosol or intramuscular route

	Animal								
Dose/Route	1	2	3	4	5	6	7	8	mean
saline control	0	0	0	0	0	0	0	0	0
10cfu/aerosol	0	3.81	3.70	4.59	2.70	5.20	0	5.80	4.30
100cfu/aerosol	4.30	ND	5.96	†	6.36	4.57	3.70	6.90	5.30
1000cfu/aerosol	4.98	4.57	4.60	4.95	6.78	†	5.00	6.95	5.40
100 cfu/i.m.	3.27	3.74	3.91	3.47	3.43				3.64

† Animal died less than five weeks after infection.

ND Not done.

Conclusion

The results of this work have allowed us to define the parameters of a low-dose aerosol challenge model (milestone 03/03) for use in all subsequent vaccine efficacy and virulence screening work. For vaccination studies, animals are routinely challenged with a dose of 10^6 cfu/ml *M. bovis* in order to obtain an inhaled retained dose in the lungs of approximately 10 organisms. This dose is referred to as 10 lfu (lesion forming units). Animals are weighed at least twice a week. When at least 50% of a given experimental group have lost 20% of their maximum recorded body weight the whole group is killed, and if appropriate, the experiment terminated. Typically for the control group this point is five weeks after infection.

Objective 04: Comparison of the virulence of two M. bovis field isolates from different geographical areas of GB using the guinea pig aerosol challenge model.

Results

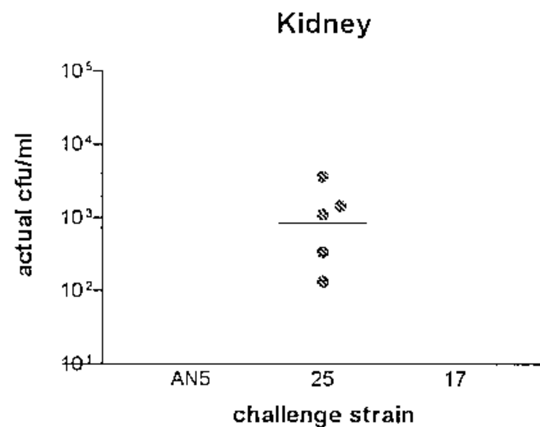
Using the challenge model described above, two field isolates of *M. bovis* were compared with the laboratory adapted strain, AN5.

Field isolates used for comparative study

AF number	Source	Spoligotype
AN5	VLA Weybridge Tuberculin Production Unit	AN5
1692/96	Cow, Hereford & Worcestershire	17
5260/96	Cow, Staffordshire	25

Guinea pigs were challenged with 10 lfu and bacterial burden in the spleen and kidney determined four weeks later. The results are summarised below:

Bacterial burden in the spleen and kidney of guinea pigs infected four weeks earlier with three strains of *M. bovis* via the aerosol route



Conclusion

Significant differences in the ability of each strain to colonise the spleen and kidney was found. No infection of the kidney was seen with either AN5 or the spoligotype 17 isolate. All strains tested were significantly from one another in terms of their ability to colonise the spleen. Using the definition of the WHO Animal Models Task Force for TB, this would equate to differences in virulence between the strains. It is of note that the spoligotype 25 isolate from a recent herd breakdown in Staffordshire was the most virulent of the strains tested. Ability of a strain to disseminate from the lung to other organs may have implications for risk of transmission. In particular, ability to colonise the kidney is likely to relate to shedding of organisms in the urine.

Objective 05: To determine the protective efficacy and tuberculin sensitisation in guinea pigs of two auxotrophic mutants of BCG

Methodology

Bacterial Strains and Propagation: M. bovis BCG Pasteur parental strain (vaccine D2) was compared with methionine and leucine auxotrophic mutants of BCG Pasteur (vaccines D10 and H10, respectively) obtained from Prof W R Jacobs, Albert Einstein College of Medicine, USA, for ability to protect against low-dose aerosol challenge with *M. bovis* and priming for a cutaneous DTH reaction to tuberculin. The challenge strain of *M. bovis* used in this study (1692/96) was isolated from a tuberculin test reactor cow in 1996 and cultured at VLA Weybridge. Cultures of BCG Pasteur and the BCG auxotrophs were grown in 50ml M-ADC-TW broth supplemented with 0.5% Casamino Acids in 490 cm^2 roller bottles at 37°C. *M. bovis* strain 1692/96 was grown as a stationary culture in 100ml M-ADC-TW broth supplemented with 4.16mg/ml sodium pyruvate. When the cells reached densities of >$5x10^7$ cfu/ml, the cells were resuspended in PBS and frozen in 1ml aliquots. (The frozen aliquots of low passage *M. bovis* strain 1692/96 are to serve as stocks for future vaccine protection experiments).

For enumeration of organisms in host tissue: BCG Pasteur was plated on Middlebrook 7H10 agar containing 0.2% (v/v) glycerol and 10% (v/v) Middlebrook OADC enrichment; the auxotrophic BCG mutants were plated on the same medium supplemented with 0.5% Casamino Acids; and *M. bovis* strain 1692/96 was plated on Middlebrook 7H10 agar containing 4.16mg/ml sodium pyruvate and 10% (v/v) Middlebrook OADC enrichment. Where necessary, serial dilution of bacterial suspensions was made in water containing 0.05% (v/v) Tween 80 to maintain dispersion.

Vaccination: Immediately prior to injection, the concentration of the BCG vaccines was adjusted to $2x10^5$ cfu/ml and the cells dispersed by brief sonication. Female Dunkin-Hartley guinea pigs weighing between 350-450g were injected subcutaneously with 250µl of each vaccine preparation (representing an inoculum of approximately $5x10^4$ cfu) in groups of six animals. Twelve control animals received 250µl of PBS each.

M. bovis Challenge: Separate groups of BCG vaccinated guinea pigs were challenged with *M. bovis* strain 1692/96 via the aerosol route five weeks after vaccination. Two doses were administered from suspensions containing approximately 10^6 or 10^7 CFU/ml in order to obtain inhaled retained doses in the lungs of approximately 10 and 100 organisms, respectively. A control group of BCG Pasteur inoculated animals were exposed to a PBS aerosol for five minutes. The weight of each animal was recorded at the time of challenge and daily from day seven after challenge. The rectal temperature of each animal was recorded at the time of challenge and weekly from then on.

Tuberculin skin testing in guinea pigs: Guinea pigs were assayed for cutaneous delayed-type hypersensitivity (DTH) five weeks after injection with the BCG vaccines. An area of approximately 10-13cm x 5cm on the flank of each animal was shaved to remove the fur. Bovine Standard PPD which contains 58,500 International Units (IU) was diluted 1:234 in isotonic PBS plus Tween to give a working concentration of 2500 IU. 0.2ml of this PPD solution was injected into the shaved dermis of each animal using a 1ml syringe fitted with a 25G needle. 24 and 48 hours later, the extent of the erythema reaction was measured using digital callipers. Two measurements were taken at right angles to one another in order to calculate the induration size in square mm.

Post Mortem examination: Aerosol infected and control animals were killed four weeks after challenge by intracardiac overdose of sodium pentobarbitone. Examination was carried out immediately after death. External examination was followed by gross internal examination of the body condition, musculoskeletal system, neck region, thoracic and abdominal cavities. The lungs with the bronchus, heart, and tracheobronchial lymph nodes attached, were removed to formal buffered saline for later examination. The whole spleen was removed aseptically and placed into 5ml sterile distilled water for bacteriology.

The fixed lungs from each animal were examined in detail and the countable lesions on the dorsal surface were recorded on a diagram of the lungs with their position and size, together with whether necrosis or consolidation was present. A numerical value representing the extent of pulmonary disease was assigned to the lungs of each animal based on the follow criteria: confluent foci where individual foci could not be enumerated reliably scored 5; foci of 3mm diameter or greater scored 3; foci of 2mm diameter scored 2; and foci of 1mm diameter scored 1. Foci smaller than 1mm were not scored. Discrete, but not confluent, foci containing necrosis scored double. The ventral surface of the lung was also examined and occasionally scored for comparison. Since

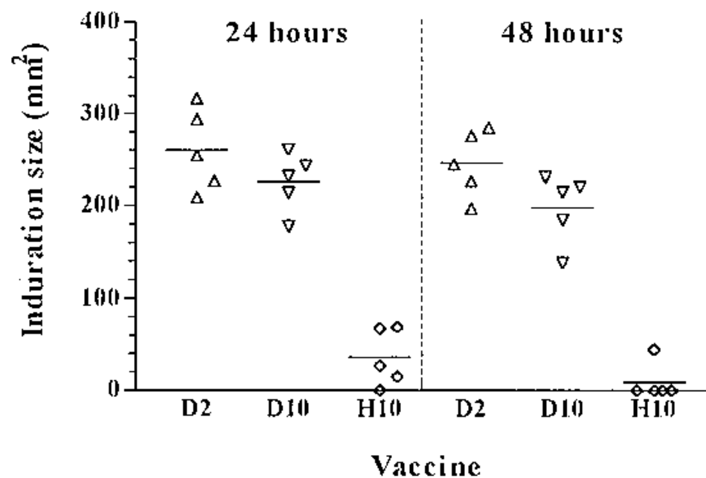
no significant differences were seen between the scores from either surface, the score from the dorsal surface was taken as representative of the whole lung pathology. The tracheobronchial lymph nodes were cut parallel to the bronchus and the width and length of each node at its widest and longest point respectively, was recorded.

Spleens were homogenised in 5ml sterile distilled water using a rotating blade macerator system. Viable counts were performed on serial dilutions of the macerate and examined after 4 weeks for growth of mycobacteria . The number of colonies on each plate containing between 30 and 300 colonies was counted and recorded.

Results

The kinetics of persistence of the auxotrophs used in this study have been examined previously in mice so were not examined in detail on this occasion in guinea pigs (objective 05/02). However, we have been unable to culture any of the BCG vaccines used in this study (D2, D10, H10) from the spleens of guinea pigs five weeks after inoculation. We have been able to culture the parental BCG from the lymph nodes draining the site of inoculation, but not the leucine auxotroph (objective 05/02). We also tested the BCG vaccines for their ability to induce cutaneous DTH in the guinea pig (Fig. 5.1). Both BCG Pasteur (D2) and the methionine auxotroph (D10) sensitised guinea pigs for cutaneous DTH reactivity. At neither 24 nor 48 hours after intradermal PPD injection was there significant difference between the induration caused by D2 or D10 (unpaired t test). In marked contrast, no significant induration was seen in the animals inoculated with the leucine auxotroph (H10). At both the 24 and 48 hour timepoint, the difference in induration between D2 and H10 sensitised animals was extremely significant ($P < 0.0001$, unpaired t test).

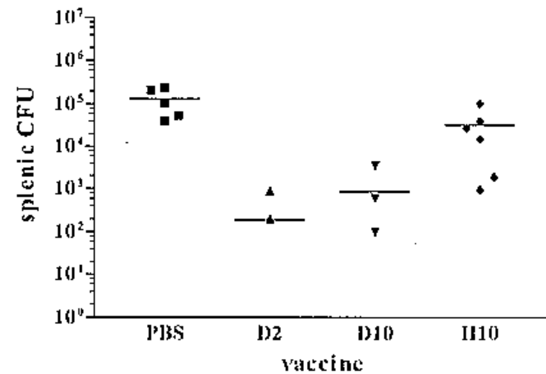
Figure 5.1



Five weeks after BCG vaccination, guinea pigs were exposed to low or moderate doses of *M. bovis* via the aerogenic route. Four weeks after aerogenic challenge, the spleens of all animals were removed and cultured for the presence of mycobacteria. Neither of the auxotrophic vaccines are able to grow on the unsupplemented medium used to culture the spleen macerate. Additionally, nothing was cultured from the spleens of control animals inoculated with BCG Pasteur (D2) and sham challenged with a saline aerosol, indicating that any colonies grown from the spleens of infected guinea pigs were solely derived from the challenge organism. All BCG vaccines conferred a degree of protection to the guinea pigs against challenge with *M. bovis* (strain 1692/96) at doses of either 10 or 100 retained organisms (Fig. 5.2A and B, respectively). The efficacy of the three BCG vaccines was related to their ability to sensitise for DTH and by inference, to their persistence. Thus, for a challenge dose of 10 *M. bovis* organisms, parental BCG (D2) gave 5.3 log protection, the methionine auxotroph (D10) gave 3.8 log protection, and the leucine auxotroph (H10) gave 2.6 log protection. Most strikingly, no organisms could be recovered from the spleen of any animal vaccinated with BCG Pasteur (D2) and challenged with the low dose of *M. bovis*. At the higher challenge dose of 100 organisms, protection was significant but less dramatic.

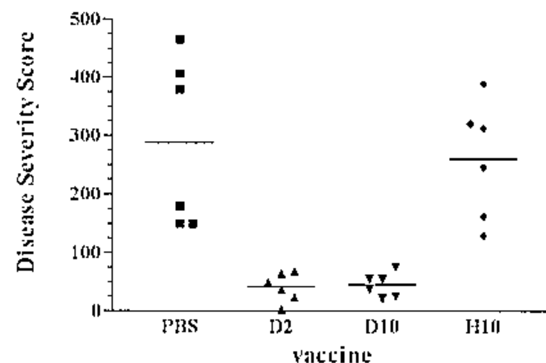
vaccine

Figure 2B



The lungs and associated lymph tissue was examined after formalin fixation and an assessment made of the gross pathology seen. No significant pathology was seen in the lungs of control animals inoculated with BCG Pasteur and sham challenged with a saline aerosol. For the remaining animals, a Disease Severity Score was determined on the basis of gross lung pathology (Fig. 3). As anticipated, the most severe pathology was seen in the unvaccinated groups challenged with 100 *M. bovis* organisms. BCG Pasteur (D2) and the methionine auxotroph (D10) gave equivalent and significant protection against pulmonary disease following challenge with *M. bovis* at either dose (Fig. 3). In fact, the significance of protection was greater at the higher challenge dose. In contrast, the leucine auxotroph (H10) conferred significant protection only to the lower dose of *M. bovis* ($P < 0.02$, unpaired t test with Welch correction). As implied by the Disease Severity Score, vaccine protection from pulmonary disease was expressed as a reduction in the number and size of lesions, and the number of necrotic lesions.

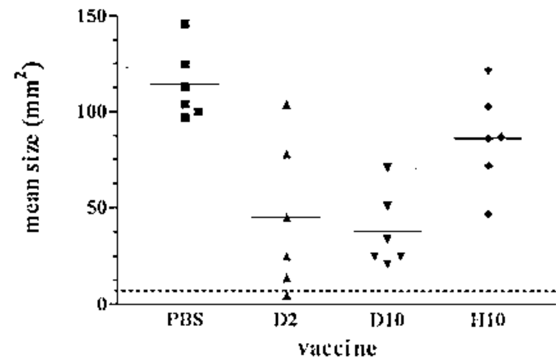
Figure 3 - 100 challenge organisms



Each tracheobronchial lymph node was measured along its two longest axes and an average value for the node(s) obtained (Fig. 4). Whereas the Disease Severity Score indicates specifically the severity of pulmonary tuberculosis, the size of the tracheobronchial lymph node reflects the extent of pulmonary disease in general. It was frequently impossible to detect the tracheobronchial lymph nodes in the BCG Pasteur (D2) immunised, unchallenged control animals (dashed line on graph), whereas the challenged animals had from one to ten discrete nodes clearly visible. The average size of the tracheobronchial lymph nodes in the control animals was $7mm^2$. D2 vaccination of the animals receiving 10 *M. bovis* organisms was the only situation where lymph node enlargement was not significantly above the control ($P = 0.38$, Mann-Whitney test). In the most extreme case (PBS vaccinated animals challenged with 100 *M. bovis* organisms), the tracheobronchial lymph node were enlarged over 15x fold; representing a volume increase in the order of 60x fold. In the animals that received 10 challenge organisms, vaccines D2 and D10 gave significant protection against *M. bovis* induced lymph node enlargement ($P < 0.024$, unpaired t test with Welch correction). In contrast, H10 did not. At 100 challenge organisms, D2 and D10 gave very significant protection against lymph node enlargement caused by *M. bovis*

infection (P < 0.006, unpaired t test with Welch correction). Again, H10 did not significantly influence lymph node enlargement.

Figure 4 - 100 challenge organisms



Objective 06: To complete vaccine protection studies for Ag85 and MPB83 DNA vaccines in guinea pigs.

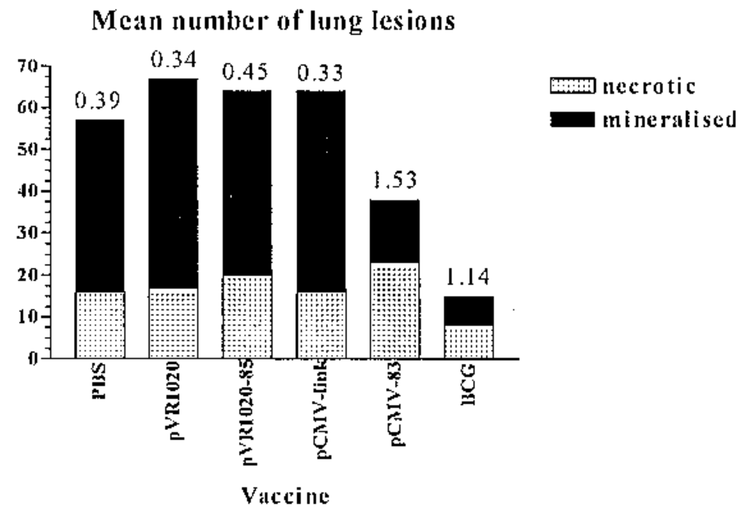
Methodology

We compared the protective response of two DNA vaccine candidates in the low-dose aerosol challenge guinea pig model already described. DNA constructs encoding the TB-complex antigens Ag85 (pVR1020-85) or MPB83 (pCMV-83) were used along with their respective control plasmids (pVR1020 and pCMV-link). Protective responses were compared with immunisation with PBS or the 'gold standard' vaccine, BCG Pasteur. Groups of six guinea pigs were immunised with each of the vaccines together with a PBS control group. The DNA immunised groups received 100µg DNA intramuscularly into each hind leg on two occasions, three weeks apart. The BCG group received $6x10^4$ cfu subcutaneously once at the time of the second DNA immunisation. Eight weeks after the final immunisation, all animals received aerosol challenge with 10 retained organisms of *M. bovis* strain 2122/97. Temperatures were measured weekly and body weight daily to indicate signs of distress. Animals were sacrificed after 10 weeks (or sooner if distressed) and *post mortem* examination carried out. Lungs were fixed in buffered formalin for detailed examination, histology, and X-ray. Spleens were taken for subsequent bacteriology.

Results

Guinea pigs immunised with pCMV-83 had 33% fewer lesions in their lungs 10 weeks after low-dose *M. bovis* aerosol challenge but were not protected from haematogenous seeding of the spleen. The number of necrotic and mineralised pulmonary lesions was determined by X-ray and examination of fixed lungs *post mortem*. Lesions of control animals were predominantly mineralised. The opposite was seen in pCMV83-immunised animals, where pulmonary lesions were predominantly necrotic. Compared with controls, BCG-vaccinated animals had an average of 1.4 log fewer *M. bovis* in their spleen (no bacteria were recovered from the spleens of 3/6 animals), and 74% fewer pulmonary lesions; of which equal numbers were mineralised and necrotic. Vaccination with pVR1020-85 gave no protection in the lung or spleen, and had no influence on the proportion of mineralised : necrotic lesions.

10
PB
pVR102
pVR1020-8
pCMV-lin
pCMV-8
BC
Vaccine



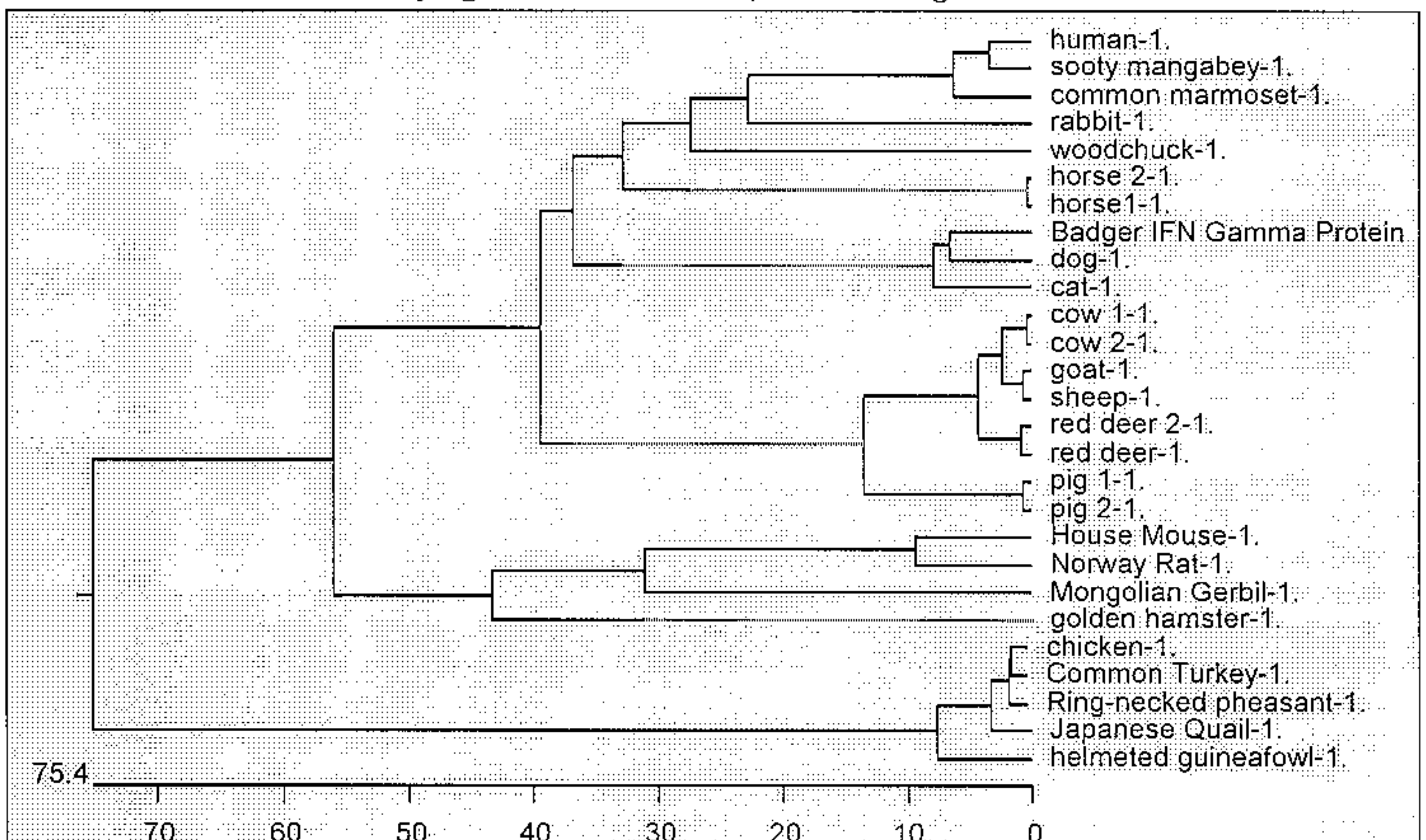
Conclusion
While DNA vaccination appears to have little effect in controlling the numbers of organisms which spread from the lung to the spleen, DNA vaccination of guinea pigs with MPB83 but not Ag85 appears to reduce the pathology of the disease within the lung.

Objective 07: To sequence the gene encoding badger gamma interferon.
Methodology & Results
Messenger RNA was extracted from ConA stimulated badger lymphocytes using a guanidine isothiocyanate/acid-phenol method, then precipitated and washed according to the method by Chromzynski and Sacchi.
The 3' sequence of badger IFNγ cDNA was obtained by 3' Rapid Amplification of cDNA ends (3' RACE) as follows. An Oligo (dT) Primer was used to prime first strand DNA synthesis catalysed by AMV Reverse Transcriptase, followed by digestion of contaminating mRNA with RNAseH. PCR amplification of the 3' badger IFNγ cDNA sequence was achieved using a Universal Amplification Primer (UAP) to the 5' end of the cDNA, with a Gene-Specific Primer based on a region of sequence consensus derived from human, cattle and mouse IFNγ. The resultant PCR product was cloned into plasmid pAMP1-3' and sequenced to obtain the first badger-specific IFNγ cDNA sequence. By comparison with published IFNγ sequences the badger IFNγ sequence was closest by DNA and amino acid homology to canine IFNγ. Furthermore, we deduced we had cloned the whole of the badger IFNγ cDNA except for ~100bp at the 5' end.
In order to obtain the missing 5' sequence, 5' RACE was attempted as follows. First strand cDNA was synthesised from mRNA using a specific forward primer derived from badger IFNγ sequence, followed by digestion of contaminating mRNA with RNAseH. The cDNA product was purified and tailed with dCTP. PCR amplification of the 5' badger IFNγ cDNA sequence was achieved using an Anchor Primer to the $(dC)_n$-tailed 3' end of the cDNA, with a Gene-Specific Primer towards the 5' end of the cDNA. The resultant PCR product was cloned into plasmid pAMP1-5' and sequenced with the intention of obtaining the remaining IFNγ cDNA sequence. However, the cloned cDNA remained truncated at the 5' end despite numerous attempts to obtain the full 5' sequence by RACE. Since we were never able to obtain sequence proximal to the last 8 bases of exon 1, we conclude that the 5' end of the cDNA is inherently unstable. Interestingly, the 5' sequence of canine IFNγ has not been published and may suggest further similarity between the two sequences in this respect.
An alternative strategy was then adopted in order to obtain the 5' badger IFNγ sequence from genomic, rather than complementary DNA. Multiple sequence alignment was performed on the published genomic sequence immediately upstream of the start codon of the IFNγ gene from horse, cat, goat, and rat. A set of semi-redundant 31 nucleotide forward primers were produced based on this alignment. A genomic PCR was performed using this mix of forward primers with a reverse primer 7 base pairs from the 5' end of exon 2. By this approach we obtained a PCR product of the anticipated size (1.4kb - intron of 1238bp. plus 245bp of coding region). The product was cloned and sequenced. On the basis of this sequence, primers were designed to amplify the whole of

exon 1 and part of the 5' exon 2 sequence from genomic DNA. A nested PCR was then performed to obtain the contiguous sequence representing the 5' end of the cDNA. By further cloning and nested PCR we obtained the full-length badger IFNγ cDNA sequence (milestone 07/04).
The full-length badger IFNγ cDNA sequence has been submitted to the EMBL nucleotide sequence database (accession number Y11647). Multiple protein alignments alongside the predicted amino acid sequence of badger IFNγ have enabled us to construct the following phylogenetic tree (see below). In the absence of other mustelid IFNγ sequences, the badger protein shares most homology with canine and feline IFNγ. In the case of badger versus canine IFNγ, 132/149 (88%) amino acids are identical and 141/149 (94%) are similar.

In order to raise monoclonal antibodies to badger IFNγ (milestone 07/05), the cDNA was cloned into the mammalian expression vector, pCMV-link. The IFNγ containing clones were identified by a BamHI and XbaI digest and confirmed by sequencing. The plasmid containing the IFNγ cDNA (pCMVγ) was grown up in bulk, extracted and used to inoculate BALB/c mice. To date, an ELISA using an anti-mouse immunoglobulin antibody has detected a rise in titre over the course of the immunisation period which suggests the mice have been immunised (both primed and boosted). In order to facilitate screening of the mouse hybridomas, the cDNA has been cloned additionally into two different His-tag prokaryotic expression vectors, pTrcHis, and pRSET for the expression of the recombinant protein in *E. coli*. Once this material is obtained we shall perform the B-cell fusions on these mice.

Phylogenetic Tree of IFNγ Protein Alignments



Objective 08: To purify Badger IgA.

Methodology

Bile from culture negative badgers (obtained from badger removal operations, in Gloucestershire) was clarified by centrifugation. The clarified bile was concentrated 5-fold using a stirred cell Amicon concentrator, then dialysed 3 times in 2% NaCl ,buffered with 0.02M Tris-HCl pH 8.0 containing 0.1% Kathon. The dialysed concentrate was subjected to further centrifugation before application to a BioSep Sec 3000 Gel Filtration column, equilibrated with 50mM Na PO_4 plus 0.15M NaCl. Fractions were collected according to absorbance 280nm., and then analysed by SDS-PAGE, followed by silver stain and Western blot.

Fractions corresponding to three main peaks detected by the UV-monitor were run on SDS-PAGE mini-gels and transferred onto nitrocellulose membranes. The blots were probed with a panel of commercial (and one non-commercial) anti IgA antibodies to establish any cross-reactivity to badger IgA (milestone 08/05). The non-commercial antibody (a rabbit anti-dog IgA polyclonal) was a kind gift of Dr. Stuart Carter, Department of Veterinary Clinical Science and Animal Husbandry, University of Liverpool.

Results
The non-commercial anti-dog IgA blotted 3 bands, corresponding to the largest peak on the UV monitor trace, at 27 kDa and 66-97 kDa and an additional faint band at 46 kDa establishing that we had successfully purified badger IgA (milestone 08/04). A commercial rabbit anti-dog IgA (Bethyl) detected three bands of the same size. The remaining anti-species antibodies were rabbit anti-pig IgA (Bethyl), goat anti-cat IgA, pig anti-goat IgA and sheep anti-human IgA (Accurate). These antibodies all detected the 27 kDa and the 66-97 kDa bands. A rabbit anti-secretory component polyclonal antisera (Accurate) detected the 66-97 kDa band only, indicating that this size band, also seen with the other antibodies, corresponds to the secretory component of badger IgA. No badger IgG was detected in the badger bile by western blot using the VLA anti-badger IgG monoclonal antibody (CF2). Comparison of the badger results with published data on IgA purified from other species, indicates that the 27kDa band corresponds to the IgA light chain; the 46 kDa band, the heavy chain; and the consistently detected band at 66-97 kDa, the secretory component. The results are summarised below.

	Recognition of badger IgA		
species specificity of anti-IgA antibody	**27kDa (light chain)**	**46kDa (heavy chain)**	**66-97kDa (secretory component)**
dog	+	+	+
pig	+	-	+
cat	+	-	+
goat	+	-	+
human	+	-	+
secretory component	-	-	+

Production of an in-house monoclonal antibody to badger IgA (milestone 08/06) was considered low priority, given the wide range of commercial antibodies that successfully detected badger IgA. However, purified IgA is available and we intend to use this to immunise mice in the near future.

Objective 09: To complete DNA vaccination and protection studies in mice.
Previous work by us and others has established the immunogenicity of DNA constructs expressing MPB83, MPB70, and Ag85A when injected intramuscularly into mice. Work done as part of SC0051 showed that MPB83 was considerably more immunogenic than MPB70, resulting a rapid antigen-specific seroconversion with a mixed IgG1/2a profile. Completion of milestone 06/01 before the start of objective 09 permitted us to establish that Ag85A DNA was not protective in a guinea pig challenge model of *M. bovis* infection, whereas protection was seen with MPB83 DNA. For these reasons it seemed an unjustifiable use of mice to proceed with milestone 09/01. Instead we concentrated on the MPB83 DNA vaccine, pCMV83, and determined its ability to confer protection in a mouse intravenous *M. bovis* challenge model (milestone 09/02) as follows:

Methodology
Six week old specific-pathogen-free BALB/c mice in groups of five were immunised with either BCG Pasteur, pCMV-link, or pCMV83 DNA. BCG immunised mice received a single 50µl inoculum intradermally at the base of the tail at the beginning of the experiment, representing a vaccination dose of 1.75×10^5 cfu. DNA immunised mice received a 50µl inoculum (50µg) into the quadricep muscle of each hind leg (100µg total) at the beginning of the experiment and on three subsequent occasions, three weeks apart. 45 days after the last DNA immunisation, all mice were infected intravenously with 10^3 or 10^4 cfu *M. bovis* strain 5260/96. This strain of *M. bovis* was isolated from a tuberculin test reactor cow in the county of Staffordshire in 1996 and cultured at VLA Weybridge. This strain has been spoligotyped and designated type 25.

Mice were killed seven weeks after *M. bovis* challenge by cervical dislocation. Examination was carried out immediately after death. External examination was followed by gross internal examination of the body condition, musculoskeletal system, neck region, thoracic and abdominal cavities. Lungs, spleen, and one kidney (from the same side in each mouse) were removed aseptically and placed into 5ml sterile distilled water for bacteriology. The tissues were homogenised using a rotating blade macerator system. Viable counts were performed on serial dilutions of the macerate and examined after six weeks for growth of mycobacteria. The number of colonies on each plate containing between 30 and 300 colonies was counted and recorded.

Results

The external body condition of all animals was considered normal with the exception of one of the pCMV-link vaccinees which appeared emaciated. The lungs of all animals contained tuberculous lesions; the numbers of which did not vary significantly between the vaccine groups (Figure 1).

Interestingly, none of the other internal organs (spleen, liver, kidney) appeared diseased, although *M. bovis* was cultured from the spleen and kidney. The bacterial load in the organs from each animal is shown (Figure 2). The BCG Pasteur vaccinated mice had significantly less *M. bovis* in their lungs and spleen compared with the pCMV-link control group (p<0.05 and p<0.01, respectively) (Figure 2). Very significant protection was seen in the lungs and spleens of mice immunised with pCMV83 (p<0.01 for both organs) suggesting that this antigen alone can confer significant protection against infection with virulent *M. bovis* in mice. For the spleen, but not the lung, immunisation with pCMV83 gave significantly more protection than immunisation with BCG (p<0.05). Protection was also seen in the kidney. Although, the extent of protection did not reach statistical significance BCG Pasteur gave 0.5 log protection, whilst pCMV83 gave more at 1.2 log. when compared with the pCMV-link control group.

Figure 1

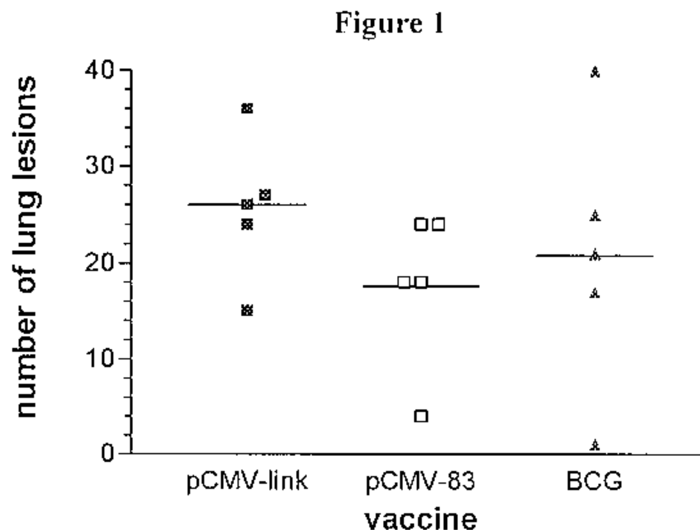
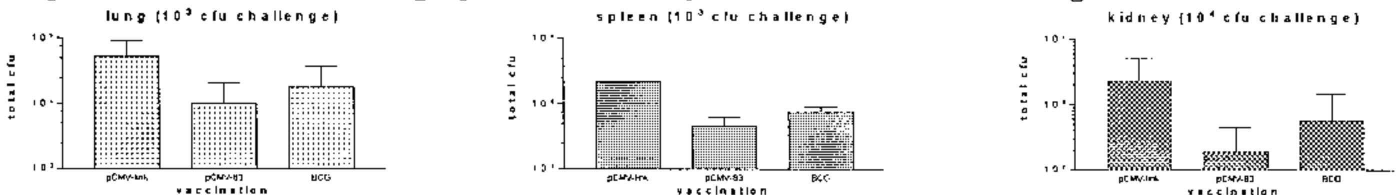


Figure 2 - total cfu in the lung, spleen ,and kidney of vaccinated mice challenged with *M. bovis* 5260/96



Objective 10: To assess the feasibility of carrying out vaccine evaluation in badgers at CNEVA, Nancy, France.

Discussions have been held with a number of Institutes including CNEVA, Abbotstown (Republic of Ireland) and Bristol University as to the feasibility of carrying out evaluation of vaccines in badgers. While initial responses have been favourable, further planning and discussions are required.

Objective 11: Development of assays to study the immunology of guinea pigs

11.1. Tumour necrosis factor-alpha (TNF-α)

A bioassay which has been described to detect human and murine TNF-α in culture supernatants has been adapted to detect guinea pig TNF-α. This is based on the on the TNF-α dose -dependent killing of the murine TNF sensitive fibroblast line L929.

11.1.1. Induction of TNF-α in macrophages isolated from guinea pig spleens:

Spleens were prepared aseptically from non-infected guinea pigs (Duncan-Hartley, ca. 400 g weight). Single cell suspensions were obtained and the cell titre adjusted to 10×10^6 cells/well with TCM (RPMI1640, supplemented with 5 % FCS, antibiotics, non-essential amino acids, 0.05 M 2-mercaptoethanol). 0.5 ml/well of this suspension was dispensed into 24 well plates and incubated for 2 h at 37 °C in a CO_2 incubator. Nonadherent cells were then washed away with warm TCM (3 times). Antigen solutions were then added in 1 ml aliquots/well and plates cultured for 24 h. Supernatants were then harvested and stored at -80 °C until testing in the L929 assay.

Antigens: LPS (5 µg/ml) and sonicates from *M. bovis* of different spoligotypes as well as AN5 diluted to 0.013 OD_{280} U/ml).

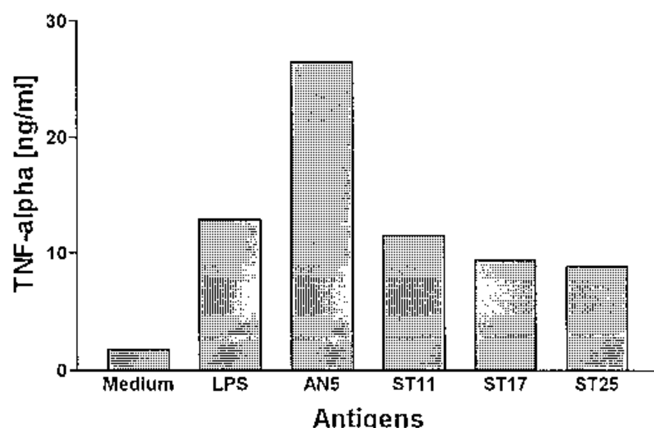
11.1.2 L929 Bioassay:

This assay was performed as described (Sheehan, K. et al., J. Immunol. 1989, 142, 3884). Briefly, 4×10^4 L929 cells were seeded per well of 96-well plates. Cells were left to adhere for 30 min, after which test samples and standard dilutions were added (100 µl/well). Recombinant mouse TNF-α was used to establish a standard curve. After an additional 30 min, 20 µl/well of actinomycin D (10 µg/ml) was added. The plates were then incubated overnight at 37 °C in a CO_2 incubator. Next morning, viability of the cultures was determined by staining with crystal violet by establishing OD_{570} in an ELISA reader. Amounts of TNF-α induced were established by comparison with the standard curve.

11.1.3. Results:

We were able to detect guinea pig TNF-a using the L929 bioassay and our results indicated that sonicates from all three *M. bovis* strains of different spoligotype (ST11, ST17, ST25) induced TNF-α in guinea pig macrophages at levels comparable to those induced with LPS stimulation. Interestingly, stimulation of guinea pigs splenic macrophages with a sonicate from AN5, the commonly used laboratory strain of *M. bovis*, induced about twice the levels of TNF-α induced with LPS or the other *M. bovis* sonicates (figure 1).

Figure 1. TNF production by guinea pig macrophages stimulated with mycobacterial sonicates



11.2. Mv1Lu Bioassay to detect guinea pig TGF-beta

A bioassay which has been described to detect human and murine TGF-β in culture supernatants has been adapted to detect guinea pig TGF-β. This is based on the on the TGF-β dose-dependent growth inhibition of the murine TGF-β sensitive mink lung line Mv1Lu. The amino acid sequence of TGF-β is highly conserved (> 95%) between mammalian species and we therefore hypothesised that this assay would also suitable to detect guinea pig TGF-β.

11.2.1. Induction of TGF-β in macrophages isolated from guinea pig spleens:

Spleens were prepared aseptically from non-infected guinea pigs (Duncan-Hartley, ca. 400 g weight). Single cell suspensions were obtained and the cell titre adjusted to 10 x 10^6 cells/well with TCM (RPMI1640, supplemented with 5 % FCS, antibiotics, non-essential amino acids, 0.05 M 2-mercaptoethanol). 0.5 ml/well of this suspension was dispensed into 24 well plates and incubated for 2 h at 37 °C in a CO_2 incubator. Nonadherent cells were then washed away with warm TCM (3 times). Antigen solutions were then added in 1 ml aliquots/well and plates cultured for 24 h. Supernatants were then harvested and stored at -80 °C until testing in the Mv1Lu assay.

Antigens: LPS (5 µg/ml) and sonicates from *M. bovis* of different spoligotypes as well as AN5 diluted to 0.013 OD_{280} U/ml).

11.2.2 Mv1Lu Bioassay

This assay was performed as described (). Briefly, 2 x 10^4 Mv1Lu cells in Iscove's modified Dulbecco's medium supplemented with 2 % FCS, L-glutamine and antibiotics were seeded per well of 96 well plates (100 µl/well). After overnight incubation at 37 °C in a CO_2 incubator, after which test samples and standard dilutions were added (100 µl/well). Recombinant human TGF-β1 was used to establish a standard curve. The plates were then incubated for 24 h at 37 °C in a CO_2 incubator after which tritiated thymidine (37 kBq/well) was added. Cells were harvested 6 h later and incorporated radioactivity established. Amounts of TGF-β induced were established by comparison with the standard curve (Figure 2).

11.2.3. Results:

We were able to detect guinea pig TGF-β using the Mv1Lu bioassay and our results indicated that sonicates from all three *M. bovis* strains of different spoligotype (types 11, 17, 25), as well as the AN5 sonicate induced TGF-β in guinea pig macrophages at comparable levels (figure 3). In addition, TGF-β levels induced by those mycobacterial stimulants were of levels equivalent to those induced by the positive control LPS (Figure 3).

Figure 11. 2.

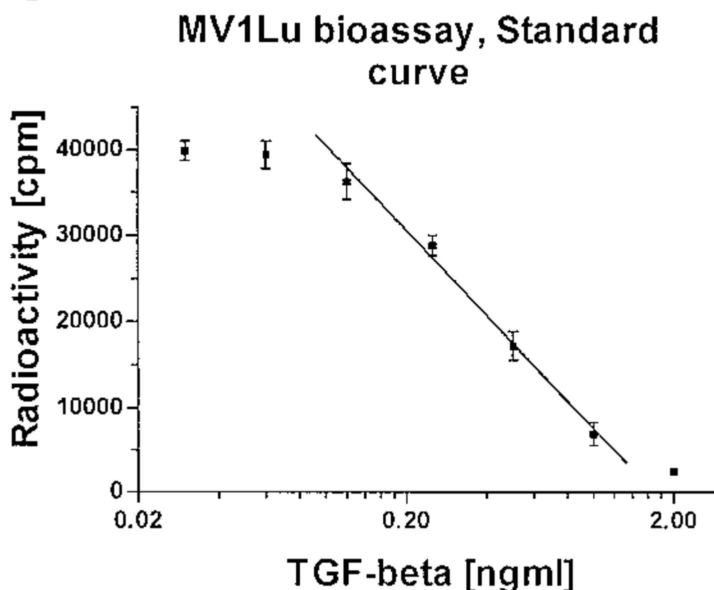
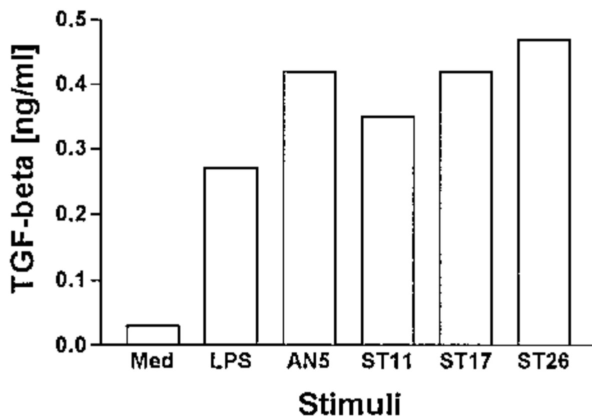


Figure 3. TGF-β production by guinea pig macrophages after stimulation with *M. bovis* sonicates



11.3. Development of RT-PCR to detect guinea pig Interferon-gamma (IFN-γ) and elucidation of the partial nucleic acid sequence of guinea pig IFN-γ.

Interferon-gamma is considered to be the most important lymphokine produced in the course of mycobacterial infections. It is therefore important to correlate IFN-g responses with protection data and also with DTH data obtained using the most important small animal model of tuberculosis, the guinea pig. However, no reliable assay to detect this cytokine in guinea pigs has been described to date. We have therefore designed PCR primers from conserved regions of IFN-g and developed an RT-PCR assay. As source of guinea pig IFN-γ mRNA we stimulated spleen cells from uninfected guinea pigs with Concanavalin A (5 mg/ml) and prepared mRNA 24 h later. This assay has been used by one of our collaborators and a manuscript is now ready for submission:
Klenner, T, Schaeffer, H, Bartels, T, Vordermeier*, M, Burger, R. Immune Reactions of CD4- and CD8-Positive T-Cell Subpopulations in Spleen and Lymph Nodes of Guinea Pigs after Vaccination with Bacillus Calmette Guerin. To be submitted to Infect. Immun.
Affiliation of collaboratiors: Robert Koch-Institut, Dept. of Immunology, Berlin, Germany and Veterinary Laboratories Agency,New Haw, Addlestone, United Kingdom*

In addition, we sequenced the PCR product and were able to obtain a partial DNA sequence which has been submitted to the database (see below for submission details).

11.3. 1. RT-PCR to detect guinea pig Interferon-gamma:

Primers:
5': AGA GCC AAA TTG TCT CCT TCT AC

3': GAG TTC ATT GAT RGC TTT GCG CTG GA

R= A,G,T,C (the original primer was degenerate at that position, but T should be the right nucleotide according of the sequencing).

PCR conditions:
Reaction mix (1 reaction): 10x buffer 5 ml
25 mM $MgCl_2$ 4 ml
dNTPs (5mM) 2 ml

Primers	2 ml each primer (Primer stocks at: 1mM)
H_2O	30.5 ml
Taq polymerase	0.5 ml
cDNA	4 ml (adjust as necessary)

Cycling conditions:

Cycle			
1		96 °C	120 s
2 -	40	96 °C	90 s
		50 °C	90 s
		72 °C	120 s
41		72 °C	600 s

The size of the PCR product is 205 bp.

11.3. 2. Partial sequence:

We have submitted the sequence to the database, although it is embargoed and will not be released until October 1999. Below are the details of the submission:

GenBank flat file:

DEFINITION Cavia porcellus interferon-gamma mRNA, partial cds.
SOURCE domestic guinea pig.
ORGANISM Cavia porcellus
Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
Rodentia; Hystricognathi; Caviidae; Cavia.
REFERENCE 1 (bases 1 to 158)
AUTHORS Vordermeier,M., Michell,S., Whelan,A., Daley,D. and Singh,M.
TITLE Direct Submission
JOURNAL Submitted (09-APR-1998) Bacteriology, VLA, New Haw, Addlestone, Surrey KT15 3NB, UK
translation="KLFEKHFTDNQTVQNSMNTIKEQIITKFFKDNSSNKVQAFKNLI
QISVNDEH"
1 caaactcttt gaaaaacatt ttacagacaa tcagactgtc caaaatagca tgaacaccat
61 caaggaacaa atcattacta agttcttcaa agacaacagc agcaacaagg tgcaggcttt
121 caaaaacctg attcaaattt cggtcaatga cgagcatg

NB. The sequence in bold is the one submitted, the primers described above are just flanking this sequence.

<u>**Objective 12**</u>

All 10 of the papers have been drafted (milestones 12/02, 12/03, 12/04).

Those published or in press:

1. A lymphocyte transformation assay for the detection of *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*). D. Dalley et al (1999) Veterinary Immunology & Immunopathology. 70, 85-94.
2. Vaccination of Mice and Cattle with Plasmid DNA Encoding the *Mycobacterium bovis* antigen MPB83. MA Chambers et al., Clinical Infectious Diseases (In Press).
3. Evaluation of the protective efficacy of BCG against aerosol challenge by *Mycobacterium tuberculosis* and *Mycobacterium bovis*. A Williams et al., Clinical Infectious Diseases (In Press).

Those submitted:

1. A Guinea Pig Model of Low-Dose *Mycobacterium bovis* Aerogenic Infection Suitable for Virulence, Vaccination, and Immunological Studies.. MA. Chambers et al., Submitted to Journal of Infectious Diseases.
2. Identification of BCG auxotrophic mutants which protect guinea pigs against tuberculosis without sensitising to tuberculin. MA. Chambers et al., Submitted to Infection and Immunity.
3. Immune Reactions of CD4- and CD8-Positive T-Cell Subpopulations in Spleen and Lymph Nodes of Guinea Pigs after Vaccination with Bacillus Calmette Guerin. T. Klenner et al., Submitted to Infection and Immunity.

The remaining four manuscripts await submission.