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Immune responses induced in cattle by vaccination with a recombinant adenovirus expressing mycobacterial antigen 85A and Mycobacterium bovis BCG
IMMUNE RESPONSES INDUCED IN CATTLE BY VACCINATION WITH A RECOMBINANT
ADENOVIRUS EXPRESSING MYCOBACTERIAL ANTIGEN 85A AND BCG

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ABSTRACT

Cattle were vaccinated with an adenovirus expressing the mycobacterial antigen Ag85A (rAd85A), with BCG followed by rAd85A heterologous boosting, or rAd85A followed by BCG boosting. BCG/rAd85A resulted in the highest direct IFN-γ responses. Cultured ELISPOT analysis demonstrated that memory responses were induced by all three protocols but were strongest after BCG/rAd85A and rAd85A/BCG vaccination.
Over the last two decades there has been a steady rise in the incidence of bovine tuberculosis (bTB) in Great Britain cattle (7) and the development of a cattle bTB vaccine is considered to be the best option for its control (7). *M. bovis* Bacille Calmette Guerin (BCG) is associated with variable efficacy both in humans and in cattle improving its efficacy is a priority (3, 6). Heterologous prime-boost strategies have been developed to improve the its efficacy (2, 8, 9, 13). In particular, application of recombinant modified vaccinia Ankara strain expressing the mycobacterial protective antigen Ag85A (Rv3804c) has shown promise in small animal models of human tuberculosis (5, 19) and human phase I clinical trials of BCG vaccination followed by MVA85A boosting are underway (9, 10).

Recently, vaccination with a replication-deficient recombinant adenovirus expressing Ag85A (rAd85A) protected mice against *M. tuberculosis* infection (12,17). To assess this viral vaccine in cattle, calves were vaccinated with rAd85A in heterologous prime-boost scenarios together with BCG to determine its immunogenicity in this natural bTB target species. rAd85A was prepared as described previously (17) and groups of five calves (ca. 6 months old, Holstein, females) were vaccinated with: 1) rAd85A (10⁹ pfu/0.5 ml, intramuscularly) at week 0, BCG Pasteur (Staten Serum Institute, Copenhagen, Denmark, 10⁶ CFU/1 ml, subcutaneously) at week 6; 2) BCG Pasteur at week 0, rAd85A at week 6), or 3) rAd85A at weeks 0 and 3. These boosting intervals were chosen because rAd85A (12, 17) and BCG (16) trigger peak responses at different times post-vaccination (1-2 weeks and 3-5 weeks, respectively), and the intention was not to boost during peak responses but at a time when effector immune responses had decreased substantially.
Peripheral blood mononuclear cells (PBMC) were prepared and cultured (14, 15) to establish the numbers of *ex vivo* IFN-γ secreting cells (spot forming cells, SFC) in ELISPOT assays (14) after *in vitro* stimulation with recombinant rAg85A (5 µg/ml, Lionex, Braunschweig, Germany), or bovine tuberculin PPD (10 µg/ml, Veterinary Laboratories Agency-Weybridge). The results, shown in Figure 1A demonstrated that all vaccination protocols resulted in the induction of T cells specific for Ag85A. Priming vaccination with rAd85A resulted in rAg85A specific responses peaking at weeks 1 to 3 in the rAd85-rAd85A and rAd85A-BCG groups (254±131 and 155±50 SFC/10^6 PBMC, respectively). Similar response levels against rAg85A were observed after BCG priming in the BCG-rAd85A group. Although BCG-induced IFN-γ response peaks may vary between experiments, the responses of these BCG vaccinated calves were not significantly different from results of earlier BCG vaccination experiments (16). In addition, the response kinetic was identical to previous results obtained after a single BCG vaccination which resulted in peak responses between weeks 3 and 5 followed by a decline in responses towards pre-vaccination levels between weeks 6 – 8 post-vaccination (16). Thus, the responses described up to week 6, i.e. immediately before boosting in the BCG prime group, are identical to those observed previously following a single BCG vaccination (‘BCG alone’ group) and were expected to further decline after week 6 if the cattle were not boosted.

Boosting BCG primed calves with rAd85A resulted in statistically significant anamnestic IFN-γ responses compared to pre-boost peak levels (Fig. 1A, 5/5 calves, mean responses, pre-boost ‘BCG alone’ peak values, week 4: 206±67 SFC/10^6 PBMC; post-boost: 914±
255 SFC/10^6 PBMC, p=0.028). This enhanced *ex vivo* response peaked one week post-rAd85A infection (week 7), and was significantly different from the other two groups at this time point (p = 0.011, compared to post-boost peak responses at week 7 in rAd85A/BCG, and week 4 in rAd85A-rAd85A). *Ex vivo* responses contracted over the following weeks (Fig. 1A). Boosting the animals in the rAd85A-rAd85A group with rAd85A at week 3 resulted in enhanced responses compared to pre-boost peak levels in 2/5 animals (mean SFC: 212±111 SFC/10^6 PBMC, Fig. 1A), peaking one week after the boost. Boosting rAd85A primed cattle with BCG resulted in enhanced rAg85A responses in 3/5 animals compared to levels at the time of boosting at week 6 (100 ± 39 SFC/10^6 at week 6, 127 ± 80 SFC/10^6 PBMC, Fig. 1A), and never exceeded IFN-γ levels observed after rAd85A priming. Responses after *ex vivo* stimulation with bovine PPD were also determined and confirmed the results obtained with rAg85A (not shown). One mechanism for the failure of BCG to recall rAd85A-primed responses, or even to induce stronger responses on its own, could be due to Ad85A-induced pre-existing immunity to BCG. A similar mechanism has been postulated to explain why exposure to environmental mycobacteria results in immunity to cross reacting antigens that limits BCG multiplication and protective immunity (1). It is also possible that rAd85A is a weak immunogen in cows, poor at priming immune responses but capable of boosting existing responses.

Measuring *ex vivo* IFN-γ responses most likely assesses effector cells and effector memory T cell responses yet recent studies of viral and parasitic infections in mice and humans have suggested that central memory responses rather than effector memory
responses correlated with pathogen clearance and protection (11, 18). However, reagents
for labelling central memory responses in cattle, e.g. bovine CCR7+ cells, are
unavailable. Therefore we developed a cultured ELISPOT system to investigate long-
term ‘central’ memory responses in cattle. Although central memory T cells have not
been formally defined in cattle, this assay has been shown to assess such responses in
other species including humans (4). Cultured ELISPOT analysis was performed 14 weeks
post-priming, when no significant \textit{ex vivo} responses were found compared to pre-
vaccination levels (Fig. 1A) by stimulating $2 \times 10^6$ PBMC/ml with rAg85 protein (2
\mu g/ml). Recombinant human IL-2 (to 10 U/ml, Sigma Poole, GB) was added to the
cultures on days 5 and 8). On days 10 and 12, half of the supernatant was replaced with
IL-2-free medium. On day 13, 2x10^4 cells/well were added to ELISPOT plates, and
incubated together with rAg85A (5mg/ml) or a Ag85A peptide cocktail (peptides
1,3,4,6,8,11,16,17,18,21,22,23 see: (16); 6 mg/ml each peptide). Large numbers of
memory cells were found after vaccination with all three protocols (Fig. 1B). However,
rAd85A-rAd85A vaccination resulted in the weakest cultured ELISPOT responses
(rAg85A stimulation: 10,650+1,270 SFC/10^6 cells) compared to BCG-rAd85A and
rAd85A-BCG (22,720+2,780, 19,380+3,430SFC/10^6 cells, respectively), although only
the responses in the BCG-rAd85A group were statistically significantly larger than those
of the rAd85A-rAd85A vaccinated calves (p < 0.05, Fig. 1B). While the cultured
ELISPOT responses observed in the BCG-rAd85A vaccinated calves were predictably
the strongest, rAd85A-BCG vaccination also resulted in re-call responses comparable to
BCG-rAd85A vaccination (Fig. 1B). Thus, despite the absence of strong \textit{ex vivo} IFN-\gamma
responses following rAd85A-rAd85A or rAd85A-BCG vaccination, all three vaccination
protocols resulted in strong re-call memory responses. In a pilot experiment, BCG-rAg85A vaccinated calves were also found to be protected from virulent *M. bovis* challenge and the extent of protection correlated positively with the level of cultured ELISPOT responses in 3 individual calves (data not shown). This is in agreement with our recent data applying recombinant fowlpox or MVA expressing the same antigen to cattle vaccination, where the BCG/MVA vaccine combination proved also to be the most immunogenic (16).

It had been reported recently that intranasal rAd85A delivery protected mice significantly against *M. tuberculosis* (17). Therefore we intranasally boosted 3 additional subcutaneously BCG-vaccinated calves with rAd85A at week 7 (10⁹ pfu/4 ml, 2 ml/nostril). BCG vaccination resulted in modest blood-based *ex vivo* IFN-γ responses to a pool of Ag85A peptides at the time of intranasal boosting with rAd85A. This response was boosted by intranasal rAd85A vaccination, peaking two weeks post-boost (Fig.2A, week 9). Enhanced Ag85A-specific central memory responses were also demonstrable by cultured ELISPOT after intranasal boosting with rAd85A (Fig. 2B).

In conclusion, the results reported here demonstrate that a heterologous prime-boost vaccination schedule based on BCG and systemic or mucosal vaccination with an adenovirus expressing Ag85A induced strong cellular immune responses in cattle and its assessment in large scale protective efficacy studies is warranted.
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REFERENCES


**FIGURE LEGENDS**

**Figure 1. IFN-γ responses after vaccination. A.** The kinetics of *ex vivo*, direct, responses after stimulation of PBMC (2 x 10^5/well) with rAg85A (5 μg/ml). Results are depicted as mean net SFC (SFC with stimulant minus SFC of medium controls) ± SEM of 5 cattle/group. *p < 0.05 compared to other two groups (one-way Anova followed by the Tukey-Kramer Multiple Comparisons post-analysis test). Circles: BCG-rAd85A (n=5); squares, rAd85A-BCG (n=5); triangles, rAd85A-rAd85A (n=5). Priming vaccination: week 0; B1: rAd85A boost of rAd85A-rAd85A group. B2, BCG or rAd85A boost of BCG-rAd85A or rAd85A-BCG group animals, respectively. Positive responses: SFC with minus SFC medium controls: > 50 SFC/10^6 PBMC and > pre-vaccination values.

**B.** Cultured ELISPOT responses established 8 weeks after booster injections. PBMC were stimulated with rAg85A and IL-2 for 13 days, and IFN-γ ELISPOT assays performed by stimulating cultured cells with rAg85A (5 μg/ml) or a peptide cocktail derived from Ag85A (6 μg/ml, each peptide). Cultures were performed in the presence of autologous macrophages as APC source. Medium control values were subtracted and data are represented as means of groups of 5 animals ± SEM. Statistical differences between groups were evaluated using unpaired, two-tailed t-tests. Differences between BCG-rAd85A and rAd85A-rAd85A did not reach statistical significance. Horizontal line: results of peptide-stimulated cultured ELISPOT results using PBMC from a group of 6 unvaccinated control animals (SFC/10^6 cells: 480±480).
Figure 2. Intranasal boosting with rAd85A results in increased *ex vivo* (A) and cultured (B) ELISPOT responses. (A). PBMC were prepared before rAd85A boost (week 7, black bar) and at week 9 (striped bar) of the experiment, and stimulated with a pool of immunodominant Ag85A derived peptides. Results are expressed as mean SFC/10^6 PBMC ± SEM (n=3). (B) SFC were determined after culture with rAg85A in the cultured ELISPOT protocol at week 7 (black bar) and week 10 (striped bar) of the experiment. ELISPOT assays were performed 13 days after culture initiation using the synthetic peptide cocktail used in A, in the absence of additional APC. Results are expressed as mean SFC/10^6 cells ± SEM (n=3).

*, p < 0.05 (unpaired t-test).
Figure 1
Figure 2