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Research Article

Replication Defective Adenovirus Serotype 5 Expressing Marek's Disease Virus Envelope Glycoprotein as a Potential Marek's Disease Vaccine in Chicken

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Abstract

The protective efficacy of a non-replicating Adenovirus serotype 5, expressing the immunogenic envelope glycoprotein B (Ad5-gB) of Marek's disease virus, was investigated in a vaccine-challenge model for Marek's disease in experimental chickens. *In ovo* vaccination with Ad5-gB, with or without a second vaccination post-hatch, was compared with pCVI988 (a clone of the gold-standard CVI988 Marek's disease vaccine). *In ovo* vaccination with Ad5-gB, without the second vaccination, gave a protective index of 37.5%, but did not reduce replication, shedding or transmission of virulent virus. *In ovo* vaccination followed by a post-hatch vaccination with Ad5-gB, was as protective as pCVI988 against mortality and Marek's disease lesions (100% protection) and, like pCVI988, efficiently reduced the level of virulent virus in the blood of chickens. However, although this double-dose Ad5-gB vaccination delayed the onset of shedding of virulent virus, it did not inhibit shedding and was less effective than pCVI988 in reducing shedding and transmission of virulent virus. Further optimisation of Ad5-gB dose, administration route and time of vaccination could lead to trials as a potential vectored vaccine candidate for Marek's disease, with a number of advantages over the current live cell-associated vaccines: no requirement for maintenance of a cold chain during vaccine preparation and administration, no horizontal spread, reduced selection pressure for highly virulent virus, and no possibility of reversion to virulence.

INTRODUCTION

Marek's Disease (MD) is a lymphoid neoplasm of chickens, caused by serotype-1 strains of Marek's disease virus, an α-herpesvirus in the genus *Mardivirus* (MDV-1, or *Gallid herpesvirus 2*) [1,2]. Intensive poultry farming is heavily dependent on vaccines to control disease. MD has been successfully controlled since the introduction of vaccination in 1970, the total annual worldwide vaccinations numbering over 20 billion. 'Classical' MD vaccine viruses are *Mardivirus* strains which are both naturally avirulent and non-oncogenic, or have been attenuated by serial passage in cell culture, and are generally administered as cell-associated live virus either to embryonated eggs or to neonatal chicks using automated vaccinating machines. Development of protective immunity requires replication and persistence of the vaccine virus. CVI988/Rispens, an attenuated serotype 1 strain of MDV [3], is the gold standard vaccine strain and is widely used singly or in combination with the antigenically-related herpes virus of turkeys (HVT) [4].

Classical MD vaccine viruses, while highly protective, have some drawbacks inherent to the biological characteristics of the virus. Firstly, since they are cell-associated live viruses, delivery of the recommended dose of vaccine requires a cold chain to maintain vaccine virus viability through storage, handling and reconstitution. This is not always practically achieved at the

hatchery [5] and is a major contributing factor to 'vaccine breaks' (excessive MD losses in vaccinated flocks). Secondly, MD vaccines do not induce 'sterilising immunity': although they protect against tumours and mortality, MD vaccines do not prevent super-infection by virulent strains of MDV, which can still replicate and be shed, albeit at a reduced level [6,7]. Vaccinated chickens, which are MDV-infected but healthy, therefore represent a source of infectious virus to naïve chickens. Finally, there is now good scientific data to back-up anecdotal reports that vaccination against MD is driving the evolution of field strains to greater virulence [8]. The introduction of each increasingly protective vaccine has been followed by the emergence of increasingly virulent field strains which are able to overcome the protection provided by that vaccine [9,10]. CVI988 is the most effective classical MD vaccine against very virulent field strains, which can break through the vaccinal protection of HVT and SB-1 vaccines. However, protection by CVI988 is now under severe pressure from increasingly virulent field strains. Despite attempts to produce more effective classical live cell-associated MD vaccines, by attenuation of highly virulent MDV strains in culture, none of these were any more protective than CVI988, and they would also be subject to the same limitations and drawbacks as existing classical MD vaccines.

There are some additional factors which may limit the efficacy of classical MD vaccines. Within the chick, replication of vaccine virus may be compromised by maternally-derived antibodies [11] which are transmitted from the vaccinated parent flock to offspring via the egg. The immune response to vaccine virus may be suppressed by environmental stresses or co-infections with immunosuppressive pathogens, and early exposure to a virulent MDV strain (prior to establishment of full vaccinal immunity) can result in disease.

The low market price of individual chickens is driving a need for MD vaccines that are cheaper and easier to produce and administer. The future of vaccination against MD may therefore require a move away from the search for new classical MD vaccines, and a move towards cell-free recombinant viral vector-based vaccine technology to overcome the short comings of classical MD vaccines. Vectored vaccines have additional advantages including: ease of production of vaccine; no requirement for cold chain for storage and reconstitution; reduced inhibition by maternally-derived antibody; ability to easily distinguish vaccine virus from pathogenic virus; no transmission to non-vaccinated chickens; and no risk of reversion to virulence.

Large DNA viruses, such as pox viruses, herpes viruses and adenoviruses, have great potential as vaccine vectors. Fowl pox virus (FPV) and HVT have already been tested as recombinant vaccine vectors for poultry. Those tested as vaccines against MD have been designed to express the immunogenic envelope glycoproteins of MDV, primarily glycoprotein B (gB) which induces virus-neutralisation [12,13]. Recombinant FPV vectors expressing gB (rFPV-gB) provided protection comparable to classical HVT vaccine, and significantly reduced the level of viremia, systemic infection, and shedding of MDV challenge virus [14]. However, some studies have shown that this level of protection was only obtained in chicks lacking maternally-derived antibody [12,14,15]. HVT vectored vaccines expressing MDV gB or gC were no more protective against MD than commercial HVT vaccine [16,17].

Adenoviruses are now extensively used as gene vectors in mammals, and they have multiple benefits [18,19]. Adenovirus vectors replicate rapidly and efficiently to high titres *in vitro*, are stable, maintain their infectivity well and are easy to prepare and purify. The genome can accommodate 2 kb of foreign DNA without significant effects on stability or infectivity, and foreign genes are expressed at high levels under the control of an appropriate promoter. Recombinant adenovirus vaccines have been developed and tested successfully in mammals, including vaccines against human immunodeficiency virus and rabies virus in man [20,21]. Vectors based on either human adenovirus (huAd) or fowl adenovirus have also proven effective, under experimental conditions, against pathogenic viruses in poultry including infectious bronchitis virus [22,23] and avian influenza [24-27]. A recombinant adenovirus expressing apoptosis-inducing proteins was recently shown to cause apoptosis when transfected into MD tumour cells *in vitro*, and may provide a basis for future methods to suppress tumours *in vivo* [28]. However, the potential of recombinant adenovirus vaccines to complement or replace classical MD vaccines has not been investigated, and we sought to investigate this possibility in the current study.

Administration of poultry vaccines via the *in ovo* route can reduce problems with early exposure to virulent MDV, by inducing earlier immunity. This route provides uniform mass vaccination, and has proven effective for early protection against MD by HVT [29]. *In ovo* administration of recombinant human adenovirus 5 (huAd5) provided protection against influenza virus infection, without affecting chick hatchability [25,27].

In this preliminary study we used an experimental vaccine-challenge model in chickens to investigate the protective potential, against MD, of recombinant huAd5 expressing MDV gB (Ad5-gB) following a first vaccination *in ovo* and a second vaccination after hatch. Protection was examined in terms of reduction in mortality, reduction of replication, shedding and transmission of virulent challenge virus, and was compared with protection provided by CVI988 vaccine.

MATERIALS AND METHODS

Vaccine and challenge viruses

The Ad5 (LPTOS)-MDV1gB (Ad5-gB) vaccine was prepared using a non-replicating human adenovirus serotype 5 (HuAd5) constructed and grown using a system based on the Vira Power Adenoviral expression system (Invitrogen) at the Vector Core facility of the Jenner Institute (Oxford, UK). The glycoprotein B (gB) gene of the very virulent MDV strain RB-1B was cloned under control of a 1.9 kb cytomegalovirus promoter (with regulatory element, enhancer and intron A), plus two tet operators and with a BGH poly (A) transcription termination sequence. The entry clone underwent recombination into the 36 kb E1- and E3-deleted pAd/PL-DEST AdHu5 genome vector (Invitrogen). This construct was transfected into 293TREx cells, and the resultant recombinant adenovirus (Ad5-gB) purified using caesium chloride gradient ultracentrifugation. The pCVI988 vaccine virus was prepared from the bacterial artificial chromosome

clone of Poulvac CVI988 vaccine (Fort Dodge Animal Health) as previously described [30] and passed 4 times in chick embryo fibroblast (CEF) cells. pCVI988 was used so that RB-1B challenge virus could be readily distinguished from RB-1B using q-PCR [31]. The very virulent MDV strain RB-1B [32] was prepared as previously described [33].

All virus stocks were quantified by titration onto monolayers of permissive cells in 6-well plates. Human embryonic kidney (HEK 293) cells on poly-L-Lysinecoated plates were used to titrate Ad5-gB. CEF cells were used to titrate pCVI988 and RB-1B. All cells were incubated at 37ºC in 5%CO_2atmosphere. At 2 days post infection (for Ad5-gB infected HEK293 cells) and 5 days post infection (for pCVI988, or RB-1Binfection) cells were washed, fixed, and stained using antibody HB3 (mouse anti MDV1-gB) as previously described [34]. Cytopathic plaques for pCVI988 and RB-1B, and single gB-expressing cells for Ad5-gB, were visualised by immunohistochemistry and enumerated to determine virus titre (as plaque-forming units [pfu] per mL for pCVI988 and RB-1B, and as infectious virus particles [vp] per mL for Ad5-gB).

Experimental birds

Maternal-antibody-free embryonated eggs, of the MD-susceptible Rhode Island Red breed, were produced from specified-pathogen-free parent flocks, maintained at The Pirbright Institute. At18 days of embryonation, eggs were divided between four treatment groups (Table 1). For *in ovo* vaccination, a small hole was punched in the egg shell above the air sac. pCVI988 (1000 pfu per dose) and Ad5-gB ($1X10^8$ vp per dose), were prepared in Dulbecco's Modified Essential Medium (DMEM) and 100µL was injected into the amniotic cavity using a 23-gauge needle. The unvaccinated control group was injected with 100µLDMEM. Sufficient eggs were inoculated to achieve eight chickens per group, allowing for a possible reduction in hatchability. The eggs were then transferred to curfew incubators at 37ºC until hatch. All chickens were identified by wing bands and each group housed in a separate positive pressure poultry isolator (Controlled Isolation Systems, USA) with access to water and a vegetable-based diet *ad libitum*. All procedures were performed under license of the UK home office. Second vaccinations (at the same doses as above) were administered intra-muscularly post hatch at one day of age for pCVI988 (the standard vaccination time for commercial pCVI988 vaccine in the field) or four days of age for Ad5-gB (to allow time for the immature chick immune system to begin to mount a memory response to the first Ad5-gB vaccination). Each vaccinated group contained eight vaccinated chickens, and three 'sentinel' chickens which were not vaccinated (either *in ovo* or after hatch). The non-vaccinated group contained 21 chickens. All eight vaccinated chickens in each group were challenged at 13 days after *in ovo* vaccination; by intra-abdominal administration of 250 pfu of virulent MDV strain RB-1Bin a 100µL volume. In the non-vaccinated group, 15 chickens were challenged. No sentinel chickens were challenged. Chickens were monitored daily for morbidity and mortality, and any reaching the humane end-point during the experiment were culled. Remaining infected and sentinel chickens were sacrificed at 49days post challenge (dpc). All culled chickens were subject to post mortem examination.

Sample collection

Every challenged chicken was blood-sampled by trained personnel at weekly intervals at 6, 13, 20, 27 and 34 days post challenge (dpc). Although the chickens were retained for a further 15 days, sample collection was not continued beyond 34 dpc, since all chickens in the control group had reached humane end-point by this time. Sentinel chickens were blood-sampled only at 28 days. Peripheral blood lymphocytes (PBL) were prepared as previously described [34]. At these same time-points, poultry dust samples were collected from each isolator. Pre-filters were removed from the isolator air exhaust, and within the isolator, the filters were shaken into a polythene bag to collect poultry 'dust'. Triplicate 5-mg aliquots of each dust sample were weighed and stored at -20ºC. In the isolators, the used filters were replaced with fresh filters.

DNA preparation and real-time q-PCR

DNA extractions from PBL and 5-mg dust samples were performed using DNeasy blood and tissue kit (Qiagen) according to the manufacturers' instructions. RB-1B challenge virus was quantified by detection of the U_s2 gene (which is absent from both Ad5-gB and pCVI988 vaccines). Real-time q-PCR primers and probes to amplify the MDV-1 U_s2 gene [35], and the chicken *ovo* transferrin (*ovo*) reference gene [36], were used for absolute quantification of RB-1B virus genomes as previously described [36] using ABI PRISM® 7500 Sequence Detection System (Applied Biosystems). Standard curves, prepared using 10-fold serial dilutions of DNA from MDV-infected CEF (for gB gene and U_s2 reactions) and non-infected CEF (for *ovo* reaction) and accurately calibrated against plasmid constructs of known target gene copy number, were used to quantify pCVI988 or RB-1B genomes per 10^4PBL [36] or per µg dust [37].

Table 1: Experimental design.

Experimental Group	First vaccination			Second vaccination		
	Vaccine	Route administered	Dose (per 100µL)	Vaccine	Route administered	Dose (per 100µL)
Unvaccinated control (n=15)	Diluent only	*In ovo*	-	-	-	-
Double-dose pCVI988(n=8)	pCVI988	*In ovo*	1 X 10^3pfu	pCVI988	Intramuscular (1 day old)	1 X 10^3pfu
Single-dose Ad5-gB(n=8)	Ad5-gB	*In ovo*	1 X 10^8 vp	-	-	-
Double dose Ad5-gB(n=8)	Ad5-gB	*In ovo*	1 X 10^8 vp	Ad5-gB	Intramuscular (4 days old)	1 X 10^8vp

Statistical analyses

Analysis of variance (Minitab v16) was used to make statistical comparisons between groups for hatchability, proportion of MDV-positive chickens, and proportion of chickens with MD lesions at time of death/end of trial. Statistical comparisons of survival between groups were made using the Log-rank (Mantel-Cox) test applied to Kaplan-Meier cumulative survival curves plotted using Graph Pad Prism v6. For each group of chickens, mean values for level of RB-1B challenge virus for PBL and dust, were determined using the $\log_{10}$ transformed copy number for each individual sample. Data were back-transformed to obtain the actual values for presentation of results. The effects, on level of RB-1B, of the factors vaccine and time post challenge, were examined using general linear models (Minitab v16). Pair wise comparisons were made using Tukey simultaneous tests; p values <0.05 were considered statistically significant.

RESULTS

Hatchability

The hatchability of non-vaccinated chickens was 83.1%. The hatchability of chickens vaccinated *in ovo* was 85% for pCVI988 vaccination and 73% for Ad5-gB vaccination. There was no significant difference in hatchability between any of the three groups, at the 95% level, so it can be concluded that there were no adverse effects of in ovo vaccination.

Protection against mortality and MD lesions following RB-1B challenge

Figure (1) shows survival in the four groups of infected chickens following challenge with RB-1B, and Table (2) summarises the proportion of birds showing MD lesions at the time of death (humane end-point, or termination of the trial at 49 dpc). In the non-vaccinated group, all 15 chickens reached humane end-point during the trial and MD-associated macroscopic lesions were observed in 13 birds. Nine chickens reached humane end-point by 10 dpc ('early mortality'), exhibiting rapid onset paralysis of the neck, and frequently enlarged spleens, but no tumours. The remaining six chickens in this group reached humane end-point between 15 and 34 dpc with gross MD tumours visible. In the single dose Ad5-gB group, five chickens reached humane end-point from 24 - 49 dpc, all having gross MD tumours. The remaining three chickens survived until termination of the experiment, at which time no gross MD lesions were observed. Within the double dose Ad5-gB group and the pCVI988 group, no chicken reached humane end-point and no gross MD lesions were observed.

Each of the three vaccinated groups showed significantly higher survival following challenge, than did the non-vaccinated group ($p<0.0001$ in each comparison), but survival in the single dose Ad5-gB group was significantly lower than that in both the double dose Ad5-gB group ($p<0.01$) and the pCVI988 group ($p<0.05$). Vaccination with pCVI988 or double dose Ad5-gB (but not single dose Ad5-gB) significantly reduced the proportion of chickens with MD lesions compared with the non-vaccinated group ($p<0.0001$ in each case).

The protective index (PI) for each vaccination regime was calculated using the following formula:

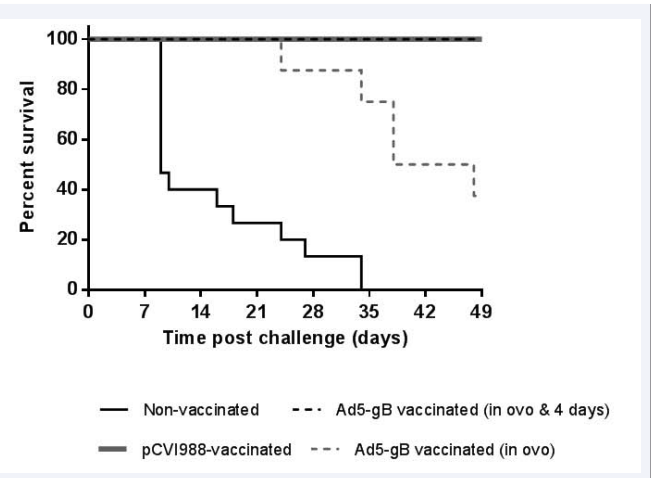

Figure 1 Ad5-gB vaccination protects against mortality following challenge with virulent MDV: Percentage survival from 0 - 49 dpc is shown for the four groups of RB-1B-challenged chickens: non-vaccinated, pCVI988-vaccinated, single-dose Ad_5-gB vaccinated, and double-dose Ad_5-gB vaccinated.

Table 2: RB-1B infection and MD lesions in challenged chickens.

Experimental Group	Proportion of chickens positive for MDV / MD in each group		
	q-PCR (PBL) (mean time to positivity for positive chickens, days)	MD lesions[a] at time of death or at end of trial (49 dpc)	Total proportion confirmed RB-1B infected by q-PCR and /or lesions
Unvaccinated control	15/15 (10.5d)	13/15	15/15
Double dose pCVI988	5/8 (24.2d)	0/8	5/8
Single dose Ad5-gB	8/8 (8.6d)	5/8	8/8
Double dose Ad5-gB	6/8 (22.2d)	0/8	6/8

[a]Enlarged spleens with / without tumours; tumours in heart, liver, kidney; swollen combs and legs

PI = 100 x (% mortality in unvaccinated challenged group - % mortality in vaccinated challenged group) / % mortality in unvaccinated challenged group [29]. Both the double dose of pCVI988 and the double dose of Ad5-gB gave a PI of 100%, while the single dose of Ad5-gB gave a PI of 28%

Replication of RB-1B challenge virus in PBL

The mean level of RB-1B (genomes per 10^4 PBL), for each group at each time point, is shown in Figure (2). The proportion of chickens in each group which became RB-1B positive in the PBL (by q-PCR), and the mean time to positivity, is summarised in Table (2). Vaccination with either pCVI988 or double dose Ad5-gB (but not single-dose Ad5-gB) significantly decreased the proportion of chickens which became MDV-positive compared with the non-vaccinated group ($p<0.01$ and $p<0.05$ respectively). There were no significant differences in proportion of MDV-positive chickens between the double dose Ad5-gB group and

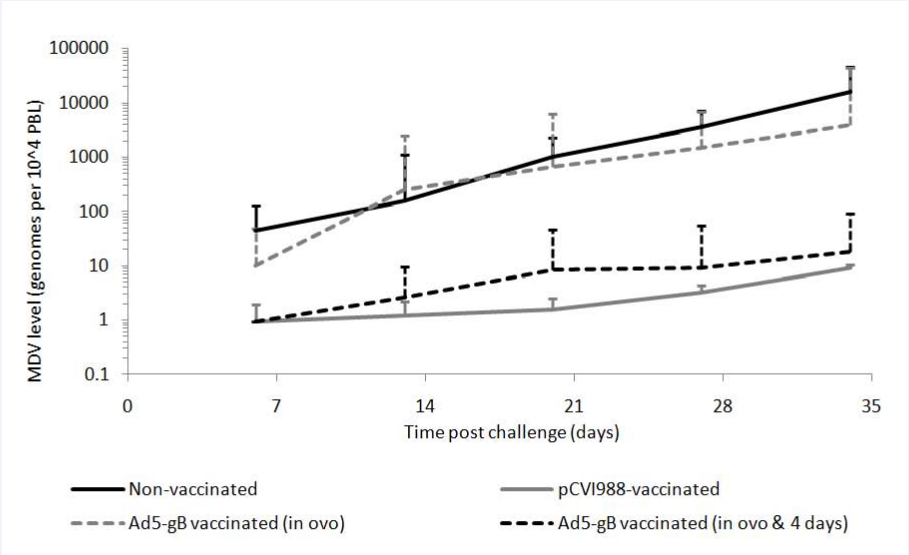

Figure 2 Ad5-gB vaccination reduces replication of virulent MDV after challenge: Mean level of RB-1B challenge virus (genomes per 10^4 PBL), from 6 – 34 dpc, for the four groups of chickens is plotted on a logarithmic scale, with 95% confidence limits (only upper confidence limit is shown for clarity).

Table 3: Transmission of RB-1B to sentinel chickens.

Experimental Group in which sentinels housed	Proportion of sentinels positive for MDV / MD in each group		
	q-PCR (PBL) at 28 dpc of co-housed challenged chickens	MD lesions at 49 dpc of co-housed challenged chickens	Total proportion confirmed RB-1B infected by q-PCR and /or lesions
Unvaccinated control	4/4 [a]	6/6 [b]	6/6
Double dose pCVI988	0/3	0/3	0/3
Single dose Ad5-gB	3/3	0/3	3/3
Double dose Ad5-gB	2/3	0/3	2/3

(a) 2/6 sentinels in this group not tested by q-PCR
(b) Enlarged spleens with visible lymphoid lesions

either the single dose Ad5-gB group or the pCVI988 group, but the difference between the pCVI988 group and the single dose Ad5-gB group approached significance (p=0.06).

Time post challenge had a significant effect on level of RB-1B in PBL (p<0.0001) with all treatment groups showing an increasing level of RB-1B from 6-34 dpc. Vaccination had a significant effect on level of RB-1B in PBL (p<0.0001). The non-vaccinated control group and the single dose Ad5-gB group had the highest virus loads, reaching 10^3-10^4 virus genomes per 10^4 PBL by 34 dpc, and there was no significant difference in virus load between these two groups. Vaccination with either pCVI988 or double-dose Ad5-gB significantly reduced replication of RB-1B compared with the non-vaccinated and single-dose Ad5-gB groups (p<0.0001), with the maximum level reached during the sampling period being <10 virus genomes per 10^4 PBL (at 34 dpc). There was no significant difference in the RB-1B level between the pCVI988 and double-dose Ad5-gB groups.

Shedding of RB-1B challenge virus

The mean level of RB-1B measured in the collected poultry dust (genomes per µg dust), for each group at each time point, is shown in Figure (3). Since the isolator filters were replaced with clean filters after each dust collection, each point represents the amount of RB-1B measured in the dust since the filter was last replaced. Time post challenge had a significant effect on level of RB-1B in dust (p<0.0001), all treatment groups showing an increase in shedding of RB-1B from 6–34 dpc. Vaccination had a significant effect on level of RB-1B in the dust (p<0.0001). The non-vaccinated control group and the single-dose Ad5-gB group shed the greatest amount of RB-1B, peaking at approximately 10^5 virus genomes per ug dust at 20 dpc, this level being maintained to 34 dpc. The level of shedding was greater in the single-dose Ad5-gB group than in the non-vaccinated group (p<0.05). Overall, vaccination with either pCVI988 or double-dose Ad5-gB significantly reduced shedding of RB-1B compared with the non-vaccinated and single-dose Ad5-gB groups (p<0.0001 in each pair wise comparison). However, although these vaccine regimes delayed the onset of virus shedding, and reduced shedding at early time-points, shedding increased with time and, by 34 dpc, the level of RB-1B detected in the dust of the double-dose Ad5-gB group was similar to that in the non-vaccinated group. Shedding of RB-1B by the pCVI988 group was significantly lower than shedding by the double-dose Ad5-gB group (p=<0.0001).

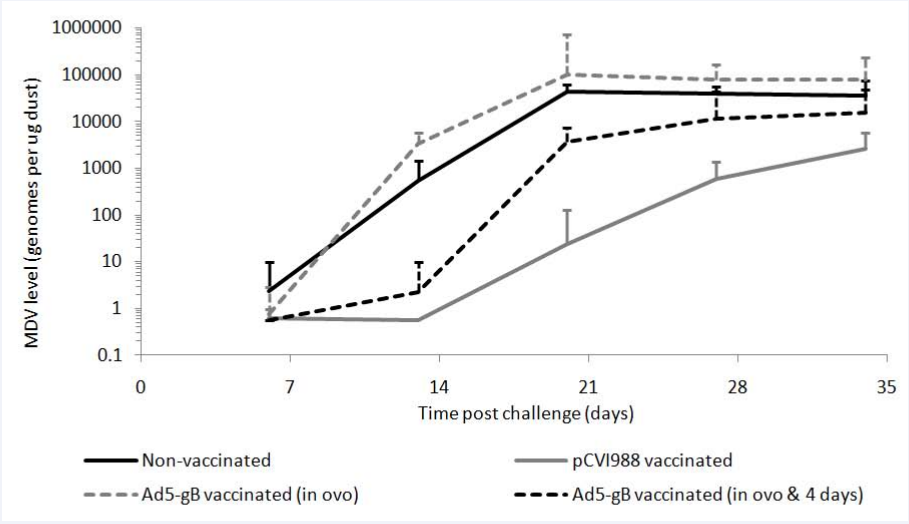

Figure 3 Ad5-gB vaccination delays and reduces shedding of virulent MDV after challenge: Shedding of RB-1B challenge virus (genomes per 1-µg dust), from 6 – 34 dpc, for the four groups of chickens is plotted on a logarithmic scale, with 95% confidence limits (only upper confidence limit is shown for clarity). Each point represents the level of RB-1B MDV in dust accumulated over the course of the previous week.

Transmission of RB-1B to sentinel chickens

Transmission of RB-1B from infected to sentinel chickens was determined by q-PCR on PBL samples from 28days, and / or by observation of MD lesions at post mortem examination (Table 3). All sentinels in the non-vaccinated group became infected with RB-1B and developed enlarged spleens with visible lesions. All three sentinels in the single dose Ad5-gB group, and two of three sentinels in the double dose Ad5-gB group were RB-1B positive by q-PCR, but no macroscopic MD lesions were observed in these chickens at post mortem examination. None of the three sentinels in the pCVI988 group became RB-1B positive by q-PCR, and no macroscopic MD lesions were observed.

DISCUSSION

Classical live cell-associated vaccines are reaching the limit of their protective efficacy against MD, as increasingly virulent field strains evolve. We examined the protective efficacy of a replication-defective human adenovirus expressing the gB gene of MDV-1 (Ad5-gB), in an experimental MD vaccine-challenge model in chickens. We sought to maximise the protective potential of Ad5-gB by giving a first vaccination *in ovo*, and a second vaccination post-hatch. Compared with our control vaccine pCVI988 (a clone of the commercial CVI988/Rispens vaccine), Ad5-gB was as protective against mortality and MD lesions (100% protection) and, like pCVI988, efficiently reduced the level of virulent virus in the blood of chickens. This confirms that the gB antigen was correctly processed, and expressed in sufficient quantity to induce a good immune response. However, although Ad5-gB vaccination delayed the onset of shedding of virulent virus, it did not inhibit shedding and was less effective than pCVI988 in reducing shedding and transmission of virulent virus to naïve sentinel chicks. Thus, like the classical MD vaccines, Ad5-gB did not induce sterilising immunity, showing that use of vectored vaccines carrying MDV genes may not remove the risk of selection for more virulent MDV field strains.

Nevertheless, Ad5-gB has a number of important advantages and potential advantages over classical MD vaccines, namely: ease of production of vaccine; no requirement for storage in liquid nitrogen; can be stabilised for storage without refrigeration [38], inhibition by maternally-derived antibody is less likely; ability to easily differentiate vaccinated chickens from chickens infected with virulent MDV; no transmission to non-vaccinated chickens; no risk of reversion to virulence; and the opportunity to insert additional genes from other avian pathogens to create a polyvalent vector vaccine. These advantages are discussed in more detail below.

Classical MD vaccines are produced by culture in primary avian cells, and subsequent harvest of these cells. Primary cells must be freshly produced from chicken embryos or young birds for each batch of vaccine, this being very time-consuming and using many animals. In contrast, adenoviruses can be grown in the continuous cell line HEK293, or other derivatives of that cell line, making significant time savings in cell preparation, and sparing many embryos and chickens. Furthermore, while classical MD vaccine viruses are cell-associated, requiring storage in liquid nitrogen and strict maintenance of a cold chain during vaccine reconstitution and administration [5], adenoviruses are cell-free, harvested from the culture supernatant and can be stored in solution or lyophilised at ambient temperature or 4ºC without significant loss of titre during storage, reconstitution and administration. This would markedly increase the likelihood of delivery of the correct vaccine dose at the hatchery or farm.

Vector-based vaccines can circumvent the problems of vaccination in the presence of high levels of maternally-derived antibody (MtAb). Chickens in commercial breeder flocks and layer parent flocks are vaccinated against MD and, at hatch, the offspring of these hens therefore have MtAb against MD, which wanes to low levels by 4 weeks of age. These antibodies can neutralise sub-optimal doses of classical MD vaccine viruses, thereby reducing vaccine replication and efficacy. MtAb against

the vector virus can be an issue for HVT and FPV-based poultry vaccines: MtAb against FPV diminished the immunogenicity of gB [39,40]. Since Ad5 is a human adenovirus, chicken populations are unlikely to have pre-existing immunity to the huAd5 viral vector and huAd5-based vaccines should therefore not be subject to interference from MtAb, and immunogenic antigens of poultry pathogens can be expressed. While classical MD vaccine viruses express a multitude of immunogenic MDV proteins, only one immunogenic protein (gB) is expressed by our vectored vaccine. Our study used experimental chicks which were free of MtAb against MDV, so it will be important to compare protection in antibody-positive chicks.

The ability to differentiate infected and vaccinated animals (DIVA) is important for immunisation strategies for any disease. For MD, DIVA is important to confirm successful vaccination and assist in identifying causes of vaccine failure, such as administration of a sub-optimal vaccine dose, interference by MtAb and infection with hyper virulent MDV field strains. Development of real-time PCR assays to distinguish between CVI988 vaccine and virulent MDV has been difficult due to limited sequence differences between them, although methods have recently been published [35]. In Ad5-gB vaccinated chickens, virulent MDV could be readily distinguished from the Ad5-gB vaccine, using PCR targeting any MDV gene other than gB.

The ability of a vaccine virus to transmit from vaccinated individuals to naïve individuals may have both advantages and disadvantages. CVI988 is readily shed from the skin of vaccinated chickens, can be detected in the environment of the poultry house, and can transmit horizontally to co-housed naïve individuals from 2-3 weeks post vaccination [41,42]. An advantage of this is that, should a proportion of the flock not be successfully vaccinated by inoculation, they may become 'vaccinated' by the transmitted vaccine virus by contact-infection from their vaccinated flock-mates. Disadvantages of vaccine virus transmission are that it may provide the opportunity for mutation of the vaccine virus and, also, circulation of sub-optimal levels of vaccine in chickens may increase the risk of evolution of more virulent field strains. Since Ad5-gB will not replicate or transmit in chickens, there is no risk of horizontal spread.

The most effective classical MD vaccines are those derived from serotype 1 strains that have been attenuated by serial passage in cell culture. CVI988 was originally an isolate of mild virulence, which was further attenuated in culture. Nevertheless, low cell culture passages of CVI988 can show some reversion to virulence causing lesions in some breeds of chicken, when used as a vaccine. Culture-passaged clones of virulent field strains may be more immunogenic as vaccines, but there is increased risk of reversion to virulence. Since Ad5-gB expresses only one MDV gene, and since Ad5 is a human virus, there is minimal risk that Ad5-gB will cause disease in vaccinated chickens.

In ovo vaccination with Ad5-gB had no significant adverse effect on hatchability, indicating that this is an appropriate option for administration of Ad5-gB. *In ovo* vaccination has a multitude of advantages compared with vaccination of the neonatal chick, enabling rapid, uniform and efficient mass delivery to large numbers of eggs using automated devices. Labour costs are reduced; there is reduced risk of contamination, and earlier stimulation of the immune response giving earlier development of protection. In this study, we vaccinated chicks with Ad5-gB *in ovo*, with or without a second vaccination post hatch. *In ovo* vaccination alone protected against early mortality following infection and delayed development of MD and it is interesting that, although slight and non-significant, the reduction of RB-1B level in the blood of Ad5-gB *in ovo* vaccinated chickens compared with non-vaccinated chickens was sufficient to fully protect against early mortality. However, the second intra-muscular vaccination was required to confer full protection against MD. It is not possible to say whether a post-hatch intra-muscular Ad5-gB vaccination alone (i.e. in the absence of *in ovo* vaccination) would have given as good protection as the two vaccinations used in this study. While this preliminary study tested only a 10^8 vp dose of Ad5-gB vaccine, it will be important for further studies to test the protective efficacy of different doses, to determine the optimum protective dose.

However, in the field where chicks may be exposed to MDV as soon as they are transferred to the poultry house, a degree of early protection by *in ovo* vaccination will be an advantage. Steitz et al. [25], also found that *in ovo* vaccination provided only limited immunity, when huAd5 was used as a recombinant vector vaccine against avian influenza. A high *in ovo* dose (10^{10} virus particles) was required to induce detectable influenza-specific neutralising antibody, and antibody levels were low and very variable between chickens. Therefore, the embryonic day on which *in ovo* vaccination is performed, and the precise site of vaccine delivery into the egg, may be important and require optimisation. Further studies are needed to investigate whether administration of a higher *in ovo* dose of Ad5-gB would provide full protection in our challenge model.

Recombinant adenovirus-based vaccines are known to induce both humoral and cellular immunity very effectively in mammals [24]. Induction of high levels of antigen-specific antibody after vaccination with recombinant huAd5 has also been demonstrated in chickens [43,25]. The sub-cutaneous (s.c.) administration route was shown to be the most effective for induction of high antibody titres in chickens [24,25]. A dose of 10^7 recombinant huAd5 virus particles was optimum: lower doses did not induce detectable antibodies, while higher doses did not increase antibody titres. The nature of the immune response and the mechanism of protection were not investigated in the current study. In further studies, vaccine-induced antibody levels should be measured in serum collected prior to challenge, and cell proliferation assays will also be required to examine the contribution of humoral and cell-mediated immunity to protection against MD by Ad5-gB. Having demonstrated effective protection against MD using an intra-muscularly-delivered dose of 10^8 virus particles in this preliminary study, it will be important for future studies to investigate the kinetics and longevity of antibody production in non-challenged chickens, following different doses and routes of Ad5-gB vaccination.

Heterologous prime-boost vaccination strategies use two different vaccines for the prime and boost, for example a DNA vaccine followed by a recombinant vaccine, two different recombinant vaccines, or a recombinant vaccine followed by a live virus vaccine [44,45]. Use of two successive vaccinations with the

same vaccine, as used in the current study, might be considered potentially detrimental, as antibodies (generated following the first vaccination) could rapidly neutralise the second dose of vaccine. However, Zeshan et al. [23], found that a recombinant adenovirus expressing the IBV spike protein significantly enhanced humoral and cellular responses in chickens vaccinated *in ovo* followed by an intramuscular inoculation after hatch. Nevertheless, it might be possible to further improve immune responses (perhaps further reducing shedding of MDV challenge virus) by an *in ovo* priming vaccination with Ad5-gB (which should not be affected by MtAb), followed by a post-hatch booster vaccination with CVI988 after MtAb levels have waned.

Double vaccination with Ad5-gB was as protective as a single dose of pCVI988 against challenge with RB-1B. However, while RB-1B is a very virulent strain of MDV, there are now strains of even greater virulence: very virulent plus (vv+) MDV strains. Commercial CVI988 gives a good level of protection even against most vv+ challenge strains. The gB gene in Ad5-gB was cloned from RB-1B and thus, in this study, the gB of the challenge virus was homologous to the gB expressed by the vaccine. Although gB is highly conserved among MDV strains, it will be important to investigate whether Ad5-gB is equally protective against heterologous field strains of MDV, and to examine whether it is as protective as CVI988 against vv+ strains. Ad5 expressing gB of a more virulent strain of MDV might be more protective against MD mortality and lesions.

There are a number of further questions to be answered before Ad5-gB could be considered as a potential commercial vaccine against MD. From the current study, the duration of protective immunity is not known, since the interval between vaccination and challenge was short. While classical live MD vaccines replicate and persist in the chicken, providing life-long protection, Ad5-gB is a non-replicating vaccine, and it is expected that expression of gB will last for only a few days. Duration of protective immunity should be tested using a range of vaccination-challenge time intervals.

In summary, we have demonstrated that a non-replicating human adenovirus expressing MDV gB is safe, immunogenic, protects chickens against MD and has several advantages over the current live cell-associated MD vaccines. Further studies are required to investigate whether Ad5-gB is a viable candidate as a new vaccine to provide sustainable protection against MD.

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